SERUM MAGNESIUM LEVELS IN GHANAIAN SICKLE CELL DISEASE PATIENTS

BY

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JULY 2015
DECLARATION

I, AGBOZO WILLIAM, author of this dissertation do hereby declare that, with the exception of references to other people’s work which has been duly cited, this work has entirely resulted from my personal original research under supervision of Dr. D. A. Antwi and Rev. Dr. C. Antwi-Boasiako and has not been presented for another degree elsewhere.

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DEDICATION

This work is dedicated to my senior pastor Rev. Dr. Charles Antwi-Boasiako and the entire membership of the Missionaries of Christ Church - Accra.
ACKNOWLEDGEMENTS

To God be the glory great things He has done. My utmost gratitude goes to God for giving me life, strength, good health and the knowledge to start and complete this work. My very special thanks go to my able supervisors Dr. Daniel A. Antwi (Head, Department of Physiology, School of Biomedical and Allied Health Sciences-University of Ghana) and Rev. Dr. Charles Antwi-Boasiako (Department of Physiology, School of Biomedical and Allied Health Sciences-University of Ghana). I will also like to appreciate the immense contribution made by Rev Emmanuel Frimpong and Miss Gifty Boatemaa Dankwah (Department of Physiology, School of Biomedical and Allied Health Sciences-University of Ghana).

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My very special thanks go to Miss Rukiya Latekaï Lartey for her support, inspiration and encouragement.
ABSTRACT

Background:
Vaso-occlusive crisis (VOC), the hallmark of sickle cell disease (SCD) is responsible for the high rate of morbidity and mortality in SCD patients with sickling of the sickle haemoglobin (HbS) being the main cause. Reduction of the cellular concentration of HbS through prevention of erythrocyte dehydration has been proposed as a possible therapeutic strategy for VOC management with the K-Cl co-transporter being a major pathway for sickle cell dehydration and subsequent sickling. Recent studies have shown that red blood cell magnesium (Mg) content modulates K-Cl co-transport activity hence it plays a key role in sickle RBC dehydration and its subsequent pathogenesis in SCD. Magnesium is a nutrient and the fourth most abundant cation in the human body. Magnesium is an essential electrolyte which is involved in over 300 enzymatic reaction and physiological functions in the body. However, Mg status in patients is frequently undetected and it is described as the most under diagnosed electrolyte abnormality in current medical practice. There are other electrolyte abnormalities in SCD such as sodium, potassium and calcium. Data on serum magnesium levels in SCD patients available worldwide are very few and conflicting making it difficult to globally or regionally generalize the outcomes of such findings as a guide or tool in clinical practice. To date, there is no data on serum magnesium levels in Ghanaian SCD patients. There is therefore, the need to gather data in that regard.

Aim: The aim of this study was to assess the levels of serum magnesium in Ghanaian SCD patients in steady state and VOC.

Methodology: The study is a cross sectional study with a total of 371 subjects. This was made up of 80 HbAA apparently healthy individuals recruited from the Accra Area Blood Centre for National blood donation, 97 HbSS SCD patients in steady state, 75 HbSS SCD patients in VOC, 56 HbSC SCD patients in steady state and 63 HbSC SCD patients in VOC recruited from the Centre for clinical Genetics at Korle Bu Teaching Hospital. Haematological profile of samples taken was measured using a lab system multiskan MS analyzer (Bioscience LTD, UK). Serum magnesium and calcium levels were determined using an Atomic Absorption Spectrophotometer (Variant 240FS manufactured by VARIAN Australia Pty Ltd). Serum sodium and potassium levels were also measured
using the Sherwood Flame Photometer (Model 420). Nitric oxide level in plasma was also measured by Griess Reagent System (ELISA Kit, Promega). Data collected was entered into SPSS software student version 20.0 and used for analyses.

**Results:**
The results from this study shows that, the mean serum Mg levels were significantly lower in SCD patients than in controls p < 0.001, however, the levels between steady state and VOC in SCD patients were not significantly different p = 0.24. The mean serum Ca level in SCD patients was significantly lower as compared with individuals with HbAA p < 0.001. Serum Na and K levels were significantly lower in SCD patients in steady state as compared to apparently healthy HbAA individual’s p < 0.001, with a further decrease during VOC p < 0.02. Serum NO levels were significantly high in HbAA individuals than in SCD patients in steady state and declined during the VOC in both the HbSS and HbSC patients’ p < 0.001. Furthermore, this study showed a weak positive correlation between serum magnesium and plasma NO levels in the HbSS and HbSC of the SCD patients in steady state r= 0.112 and r= 0.66 respectively. However there was a negative correlation between magnesium and NO levels in both HbSS and HbSC in VOC r = -0.905 and r = -1.802 repectively.

**Conclusion**
The mean serum Mg and Ca levels were significantly lower in SCD patients regardless of their clinical state as compared with HbAA individuals. Serum Na and K levels were decreased in SCD patients as compared to HbAA individuals and a further decrease during VOC. Nitric oxide levels were significantly higher in Hb individuals than in SCD patients during the steady state regardless of the genotype. Nitric oxide levels were further reduced during VOC. This study revealed a weak positive association between serum Mg and NO levels in the steady states in SCD. Generally, electrolyte levels were low in SCD patients as compared to HbAA individuals. This study confirmed electrolyte abnormality in SCD patients.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CAR</td>
<td>Central African Republic</td>
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<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
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<tr>
<td>Cl</td>
<td>Chlorine</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial Nitric oxide synthase</td>
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<tr>
<td>GMP</td>
<td>Guanosine monophosphate</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>Hb</td>
<td>Haemoglobin</td>
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<td>HbAA</td>
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<td>HbAS</td>
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<td>HbSβ</td>
<td>Haemoglobin Sβ</td>
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<td>HCT</td>
<td>Haematocrit</td>
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<tr>
<td>HPFH</td>
<td>Hereditary persistence of fetal haemoglobin</td>
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iNOS  inducible Nitric oxide synthase
K  Potassium
K+  Potassium ion
KCC  Potassium-chloride cotransporter
MCH  Mean cell haemoglobin
MCHC  Mean cell haemoglobin concentration
MCV  Mean cell volume
Mg  Magnesium
MgSO4  Magnesium sulphate
MPV  Mean platelet volume
mRNA  messanger ribonucleic acid
Na  Sodium
Na EDTA  Sodium ethylene-diamine-tetra-acetic acid
Na+  Sodium ion
NED  N-1-napthylethylenediamine dihydrochloride
nNOS  neuronal Nitric oxide synthase
NO  Nitric oxide
NOS  Nitric oxide synthase
PCT  Plateletcrit
PDW  Platelets distribution width
PLT  Platelets count
PO4-3  Phosphate ion
PP1  Protein phosphatase-1
PP2A  Protein phosphatase-2A
RBC  Red blood cell

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<td>Red cell distribution width</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCD</td>
<td>Sickle cell disease</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
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<tr>
<td>VOC</td>
<td>Vaso-occlusive crisis</td>
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<tr>
<td>WBC</td>
<td>White blood cells.</td>
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CHAPTER ONE

INTRODUCTION

1.1 Background

Sickle cell disease (SCD) is a hereditary red blood cell disorder found in populations across the world (Weatherall & Clegg, 2001). SCD is a general name for a group of inherited haemoglobinopathies in which at least one sickle haemoglobin (HbS) gene is inherited together with another type of abnormal haemoglobin (Hyacinth et al., 2010). Patients with SCD can be homozygous for the pathological haemoglobin S (HbSS) or heterozygous when HbS is co-inherited with other abnormal haemoglobins (Mehta & Hoffbrand, 2000) such as HbSC, HbSβ-thalassemia, HbSD-Punjab, HbSO-Arab and HbSE which are all subtypes of the disease (Bender & Hobbs, 2003). People with normal haemoglobin co-inherited with a sickle haemoglobin (HbS) are referred to as carriers of sickle cell trait. HbSS disease also referred to as sickle cell anaemia is the commonest and severest subtype of the disease (Piel et al., 2010). Sickle cell anaemia accounts for about seventy percent sickle cell disease patients worldwide (Modell & Darlington, 2008).

The disease afflicts about 100 million people worldwide with high prevalence in Africa and also people of the Mediterranean, Caribbean, Middle Eastern and Asia origin (Ohene-Frempong et al., 2008). About 300,000 children are born annually with SCD, making it a major public health problem on a global scale (Serjeant, 1997). Currently, the prevalence rate of SCD in Ghana is 2% of all births annually and a carrier rate of 30% in the population (Ohene-Frempong et al., 2008).

Clinical manifestations of the disease are primarily characterized by chronic anaemia, infections, haemolysis and recurrent acute vaso-occlusive crisis (VOC) (Frenette, 2002;
Ajay et al., 2014). Vaso-occlusive crisis is one unique determinant of SCD with a high prevalence, posing global threats on the affected individuals (Ajay et al., 2014). Vaso-occlusive crisis is the most frequent crisis in SCD that results in intense pains in the bones and muscles, tissue ischemia and multi-organ failure. VOC is responsible for most of the morbidity and mortality in SCD (Bunn, 1997).

The primary pathogenic event of SCD that leads to VOC is the polymerization of the mutant haemoglobin (HbS) within the red blood cells (Galkin & Vekilov, 2004). Haemoglobin S polymerization causes damage to the sickle erythrocyte membrane, among such changes is their propensity to adhere to vascular endothelium leading to likely vaso-occlusion in microcirculation (Browne et al., 1998; Kaul et al., 2009). An important feature of the HbS polymerization process and its subsequent sickling is the time the process of polymerization needs to be primed referred to as the “delay time” (Kaul et al., 2009). If the delay time for HbS polymerization is shorter than the capillary transit time, sickling will occur in the capillaries with likely vaso-occlusion and vice versa (Brugnara, 1995). Any factor that shortens the delay time will therefore accelerate polymerization and will promote microcirculatory vaso-occlusion.

Intracellular concentration of deoxy-HbS is reported to be a factor that highly influences the delay time (Cao & Ferrone, 1996). A higher deoxy-HbS concentration markedly reduces the delay time for polymer formation, thereby accelerating sickling (Brugnara, 1995). Hence, dehydration of red blood cells containing sickle haemoglobin contributes to the pathophysiology of sickling because of the high dependence of polymerization on the concentration of HbS; dehydrated red blood cells have higher intracellular deoxy-HbS concentrations.
One of the main pathways leading to red blood cell dehydration involves the efflux of K and Cl from the sickle RBC through the K-Cl co-transporter located on the red blood cell membrane (Franco et al., 1995; Gillen et al., 1996). Recent studies have shown that red blood cell magnesium (Mg) content modulates K-Cl cotransport activity hence Mg plays a key role in sickle RBC dehydration and its subsequent pathophysiology in SCD (Laires et al., 2004; De Francheschi et al., 2001). In addition, serum magnesium abnormalities have also been associated with several clinical disorders of the disease (Nduka et al., 1995) as reported by studies that investigated the role of magnesium in the pathogenesis of SCD (Prasad et al., 1999; Zemel et al., 2002).

Magnesium (Mg) is a nutrient and the fourth most important and abundant cation in the body, after Sodium (Na), Potassium (K) and Calcium (Ca). It is also the second most important and abundant intracellular cation after Potassium (Dean & Douglas, 2013). It is an essential cation that is needed for a wide variety of physiological activities in the human body. It serves as a cofactor for various physiological processes in the body by activating numerous enzymes most of which are involved in energy (ATP) metabolism, protein synthesis, muscle contraction and relaxation, normal neurological function and release of neurotransmitters, heart and lung function and nucleic acid stability. In humans, 1 to 2% of the total magnesium is stored in extracellular compartment, 67% is stored in the bone whiles 31% is intracellular (Akhtar et al., 2011). Although magnesium was considered the ‘forgotten ion’ for many years, its importance in SCD has been highlighted recently because of its role in RBC dehydration, vaso-occlusion events and endothelial dysfunction (Space et al., 2000; De Francheschi et al., 2001; Cunha et al., 2012).
Firstly, with regards to sickling of RBCs in SCD, low Mg levels in SCD is reported to be associated with abnormal activation of K-Cl cotransporter. The functional state of the cotransporter is positively regulated by a dephosphorylation reaction mediated by the enzymes: protein phosphatase-1 (PP1) and/or protein phosphatase-2A (PP2A) (Bize et al., 2003; Virgilio & Robert, 2005). An increased activity of these enzymes upregulates K-Cl transporter activity leading to cell dehydration. However, phosphorylation of the protein phosphatase enzymes (PP1 and/or PP2A) by Src family kinases (a subtype of non-receptor protein-tyrosine kinases) is reported to act as an upstream negative regulator of the protein phosphatases and that Src family kinases increased activity downregulates K-Cl cotransport activity. Activity of Src family kinases like other tyrosine kinases is in turn dependent on the binding of two magnesium ions (Hubbard, 1997). Mg therefore regulates K-Cl cotransport in vivo by enhancing Src family kinase activities and the consequent inhibition of the protein phosphatases activity then decreases K-Cl cotransport activity (De Francheschi et al., 2001). By increasing red blood cell magnesium, the abnormal activation of K-Cl co-transporter is blocked, thereby inhibiting efflux of potassium and loss of water from the cell. Again, K-Cl cotransporter is stimulated by oxidative stress and erythrocyte susceptibility to oxidative stress is reported to be moderately higher in RBCs with low magnesium levels (De Francheschi et al., 2001). Currently, magnesium salts such as MgSO$_4$ are used in clinical practice to reduce erythrocyte dehydration in SCD (De Francheschi et al., 1997).

Secondly, recent investigations have revealed that the pathophysiology of SCD is not limited to the HbS polymerization but also the adhesion of sickle erythrocytes, leukocytes and platelets to adhesion molecules on activated endothelial cells predominantly in the post capillary venules (Manwani & Paul, 2013). The endogenous potent vasodilator, nitric
oxide, is reported to inhibit such adhesions of sickle RBC, platelets and leukocytes to endothelium and also decreases expression of vascular cell adhesion molecule-1 (VCAM-1), an endothelial adhesion site implicated in sickle RBC/endothelial adherence (Space et al., 2000). Nitric oxide (NO) is a soluble gas produced by endothelial cells which is a potent vasodilator (Pearson et al., 1998) and has blood flow regulatory effect (Mack & Kato, 2006; Thomas et al., 2013). Some studies have confirmed that elevated extracellular magnesium content increases NO production (Northcott & Watts, 2004; Yang et al., 2000; Cunha et al., 2012). Pearson et al (1998) demonstrated that hypomagnesaemia selectively impaired the release of NO from coronary endothelium (Pearson et al., 1998). Interestingly, microvessels are reported to be more dependent on the Mg-induced vasodilatation by its role in increased release of NO. Magnesium therefore is implicated as an important mediator in reducing sickle cell vaso-oclusion in microcirculation (Yang et al., 2000).

In addition to these major roles of Mg in the pathophysiology of SCD reported above, elevation of extracellular Mg also results in decreased Ca levels in smooth muscle cells (Altura & Altura, 1995) which may be the primary mechanism by which extracellular Mg-induced endothelium independent relaxation is brought about (Yang et al., 2000). Low serum magnesium levels is also reported to exacerbate anemia in SCD patients (Olivieri et al., 1994; De Francheschi et al., 1997). Magnesium deficiency in vasculature is also reported to promote oxidative stress notably in endothelial cells (Wiles et al., 1997), resulting in increased reactive oxygen species (ROS) and cytotoxicity (Zhou et al., 1999). Again, magnesium plays a crucial role in the modulation of endothelial inflammation, which may be beneficial in SCD (Makis et al., 2000; Maier et al., 2004; Britain & Parise, 2007).
Magnesium has also been shown to slow clotting time, which might reduce vascular blockage and improve blood flow in SCD (Assaley et al., 1998).

Despite all these findings on magnesium’s role in SCD and in other diseases, Mg is described as the most under diagnosed electrolyte abnormality in current medical practice (Limaye et al., 2011) and the “forgotten” cation in human health (De Baaij et al., 2015). Levels of Mg are frequently overlooked in SCD (Limaye et al., 2011) even though hypomagnesaemia has been reported to be a common electrolyte imbalance in SCD (Laires et al., 2004). Despite the known frequency of hypomagnesaemia disorders in SCD, little is known about the prevalence and clinical implications of hypomagnesaemia in Ghanaian SCD patients. It is therefore important to determine the serum magnesium levels in these patients in order to guide in magnesium therapy for better management, hence the focus of this study.

1.2 Problem statement
VOC is the leading complication in SCD that renders affected individuals highest mortality and reduced working capacity globally (Bunn, 1997) and as such labelled as the hallmark of SCD (Morris et al., 2000; Belhassen et al., 2001). The development of VOC begins with the polymerization of deoxy-HbS due to dehydration that results in sickling and ends with the subsequent interactions between the sickled RBC and the vascular endothelium (Miller et al., 2000). The dehydration caused by the K-Cl cotransporter increases the HbS concentration. Magnesium inhibits the K-Cl cotransporter, decreasing K loss and thus improving the RBC hydration to prevent sickling (De Francheschi et al., 1997; De Francheschi et al., 2001).
However, magnesium levels are frequently overlooked in SCD (Limaye et al., 2011) even though it has been reported that hypomagnesaemia is a common electrolyte imbalance in SCD (Laires et al., 2004) with low RBC Mg content in SCD patients and low levels of serum Mg detected in children with SCD (Altura et al., 2002; Indonije et al., 2011). Again, the undetected hypomagnesaemia reported in SCD brings about secondary disturbances in other electrolytes such as potassium, phosphate, calcium and sodium (Soave et al., 2009; Godron et al., 2012) and this results in further and severe clinical disorders of the disease. Furthermore, Mg depletion is reported as the most under diagnosed electrolyte abnormality in present medical practice (Limaye et al., 2011) and the “forgotten” cation in human health (De Baaij et al., 2015).

Data on serum magnesium levels in SCD available worldwide is few and conflicting; some studies reported normal circulating levels of magnesium (Akenami et al., 1999; Oladiipo et al., 2005), while others reported low levels (Olukoga et al., 1990; Zehtachi et al., 2004). To date, there is no single data linking serum magnesium levels in Ghanaian SCD patients in steady states or in crisis.

1.3 Justification

Till date, clinical laboratory findings have not clearly defined reference ranges for Mg levels in SCD and its effects on disease severity. There is therefore the need for a local assessment of serum magnesium levels within any SCD population such as Ghana. Since the few data on SCD serum Mg levels available worldwide are conflicting (Olukoga et al., 1990; Akenami et al., 1999; Zehtachi et al., 2004; Oladipo et al., 2005) it is difficult to globally or regionally generalize the outcomes of such findings as a guide or tool in clinical practice.
In Ghana, considerable works have been done on the epidemiology of SCD and its complications (Edwin et al., 2011); however, data linking serum magnesium, SCD presentation and its severity are missing. Data is therefore needed to be collated in this regard, hence the focus of the current study. The current study will therefore contribute to the understanding of the pathophysiology of magnesium in SCD patients in Ghana. Again, the outcome of this study will provide information on dietary Mg supplementation and therapeutic guidelines.

1.4 Aim

The aim of this study was to assess the levels of serum magnesium in Ghanaian SCD patients.

1.5 Specific Objectives

- To compare serum magnesium, calcium, potassium, sodium and plasma nitric oxide levels in SCD patients (steady states and VOC) and apparently healthy HbAA individuals.

- To determine the association between serum Mg and nitric oxide in SCD patients (steady states and VOC) and apparently healthy HbAA individuals.

1.6 Hypothesis

There is no significant difference between the serum magnesium level in SCD disease patients and apparently healthy HbAA individuals.
CHAPTER TWO
LITERATURE REVIEW

2.1 SICKLE CELL DISEASE

2.1.1 Definition

Sickle cell disease (SCD) is a hereditary red blood cell disorder found in populations across the world (Weatherall & Clegg, 2001). SCD is a general name for a group of inherited haemoglobinopathies in which at least one sickle (S) betaglobin gene is inherited together with another type of abnormal haemoglobin (Mehta & Hoffbrand, 2000). The name of the disease is derived from the peculiar shapes that red blood cells (RBCs) acquire upon deoxygenation, morphologically defined as slender, elongated banana-like, crescent-shaped or sickle-like cells with sharp protrusions (Dorn-Beineke & Frietsch, 2002).

2.1.2 History

SCD disease is believed to have been long in existence before the first reported case in literature. Oral history passed down the generations in Africa gave account of an inherited chronic disease characterized by recurrent episodes of bone pain associated with cold. The first written account of the condition was published in 1874 by Africanus Horton. Africanus described various features of the inherited disease including persistent abnormality of blood, painful crisis associated with fever and increased frequency of the painful episodes during the rainy season (Okpala, 2004). SCD was first described in western medical literature by the American physician James Herrick who reported the presence of ‘peculiar elongated and sickle-shaped red blood corpuscles’ in the blood film of a Grenadan student in case of severe anaemia and a history of leg ulcers, shortness of
breath and jaundice (Herrick, 2001). The disease was subsequently called sickle cell anaemia.

A sustained interest of the scientific community about the disease led to a major breakthrough in 1949 when Linus Pauling and colleagues discovered that sickle cell anaemia is caused by a mutation in the gene for betaglobin (Galkin & Vekilov, 2004). In that same year, the autosomal recessive inheritance of the disease was elucidated (Frenette & Atweh, 2007). Ingram and colleagues also demonstrated shortly thereafter that the mutant sickle haemoglobin (HbS) differed from normal hemoglobin A (HbA) by a single amino acid substitution (Frenette & Atweh, 2007). So, three-quarters of a century after the original documentation by Africanus Horton, sickle cell anaemia became the first human disease to be described at the molecular level.

This was followed by studies that analyzed the structure and physical properties of HbS (Ferrone, 2004), molecular basis of the sickling phenomenon, cloning and sequencing of the betaglobin gene, development of molecular diagnostic methods and establishment of prenatal diagnosis (Chakravorty & Williams, 2015). In parallel with these advances, significant progress was also made towards improving clinical outcomes among those born with SCD during the 1970s and 1980s, before which very few affected subjects survived beyond 10 years. Currently advanced studies are ongoing, placing SCD at the leading edge of investigations to elucidate the molecular basis of human diseases (Ferrone, 2004).
2.1.3 Classification of SCD

SCD encompass a group of inherited conditions characterized by the co-inheritance of at least one betaglobin S (β⁵) gene and a second mutant variant of human β-globin gene. Patients with SCD can be homozygous for the pathological haemoglobin S (SS) or heterozygous with a co-inherited other abnormal haemoglobin (Mehta & Hoffbrand, 2000).

The most common subtype of SCD results from the homozygous inheritance of the betaglobin S (β⁵) mutation that codes for sickle cell haemoglobin (HbS) (Modell & Darlison, 2008). Homozygous SCD is also referred to as sickle cell anaemia, Hb SS or SS sickle cell disease. Sickle cell anaemia is responsible for the most common and severest variant of SCD. It accounts for 60% to 70% of sickle cell disease in the US (Bender & Hobbs, 2003). Within Africa, the frequency of Hb SS, is highest in low-altitude equatorial regions (Piel et al., 2010).

The second subtype of SCD which is common in Africa is compound heterozygosity of abnormal HbS and HbC haemoglobins. The haemoglobin C allele is found almost exclusively among people of West African ancestry, being most common among those in Burkina Faso and northern Ghana (Bender & Hobbs, 2003).

Compound heterozygosity of haemoglobin S with β-thalassemia (a mutant β-globin gene that either fails to produce normal β-globin mRNA or produces it at markedly decreased levels) is also a form of SCD that is believed to be rare in most of sub-Saharan Africa. Two types of sickle β-thalassemia exist: Hb Sβ⁺-thalassemia and Hb Sβ⁻-
thalassemia. β⁺-thalassemia occurs when reduced levels of normal β-globin chains are produced and β⁰-thalassemia occurs when there is no β-globin chain synthesis.

Other globin β chain variants such as D-Punjab, O-Arab and E also result in sickle cell disease when co-inherited with HbS (Bender & Hobbs, 2003).

People with normal haemoglobin (HbA) co-inherited with a sickle haemoglobin (HbS) are referred to as carriers of sickle cell trait (HbAS). They are not considered as SCD patients because it does not cause clinical illness. The prevalence of the carrier state (HbAS) occurs in 1 in 4 Nigerians, 1 in 5 Ghanaians and 1 in 10 Afro-Caribbeans (Powers, 1991).

2.1.4 Clinical manifestations of SCD

SCD is accompanied with various clinical manifestations due to three pathological processes: sickling of red blood cells, vaso-occlusion and susceptibility to infections. The disease is primarily characterized by chronic anaemia, sepsis, haemolysis and recurrent acute vaso-occlusive crisis (Rodgers, 1997; Frenette, 2002).

In most of these patients a chronic haemolytic anaemia accompanied with jaundice caused by a decreased mean red blood cell survival (10 to 15 days compared to 120 days in a normal individual) is found. Splenic sequestration crisis aggravate the pre-existing mild anaemia to severe low-oxygenated episodes (Dorn-Beineke & Frietsch, 2002).

In SCD, occurrence of recurrent and widespread occlusion of blood vessels deprives parts of the body of blood and nutrients resulting in ischaemia and infarction. Vascular occlusions are usually characterized by pains, systemic inflammatory responses that may be severe, episodic and unpredictable. Vaso-occlusion is responsible for the chronic multi-
organ failure in SCD, such as renal insufficiency (Warandy & Sullivan, 1998), infarction of spleen, liver, kidney and lung (Powars, 2000). It also results in neurologic complications (stroke, silent cerebral infarcts, cerebral haemorrhage, cerebral blood flow abnormalities) (Bernaudin et al., 2011), cutaneous leg ulceration, priapism, acute chest syndrome (Bakanay et al., 2005), skeletal changes, pulmonary (Minter & Gladwin, 2001) and splenic dysfunction (Dorn-Beineke & Frietsch, 2002) and retinopathy (Mehta et al., 2001).

Again, SCD patients are faced with proneness to infections because of hyposplenism (Anyaegebhu et al., 1999). Intercurrent infections particularly of respiratory tract, fever, abdominal pain, skeletal pain, haematologic and bone pain crisis are the main causes of morbidity in sickle cell patients (Ibe et al., 2009) and the leading cause of mortality in SCD (Okpala, 2004).

Specific symptoms of SCD occur at different ages. Newborns are protected by the high intraerythrocytic haemoglobin F (HbF) content (HbF level >20% protects sickling). After the first few months of life, as βS-globin production increases and HbF declines, the clinical syndrome of SCD emerges. One of the first symptom which occurs is the symmetric, painful swelling of hands and feet (hand-foot-syndrome, dactylitis) caused by vascular occlusion of extremity end arteries (Gill et al., 1995; Dorn-Beineke & Frietsch, 2002).
2.1.5 Sickle Cell Crisis

Sickle cell crisis is defined as any sudden deviation for the worse that will not have happened had the patient been without the sickle cell gene (Konotey-Ahulu, 1991). Implicit in the definition are elements of unpredictability, suddenness and clinical worsening of the condition of the patient. Pathophysiologically, the crisis can be considered as the onset of acute symptoms due generally to sudden in vivo sickling. It is never without a precipitating cause (Konotey-Ahulu, 1991). There are four types of sickle cell crisis some of which are painful, and these include; sequestration crisis, haemolytic crisis, aplastic crisis and vaso-occlusive crisis (Konotey-Ahulu, 1991). Vaso-occlusive crisis will be considered alone in this study.

2.1.6 Vaso-occlusive crisis (VOC)

Although clinical manifestation of SCD displays a wide array of symptoms, recurrent attacks of vaso-occlusive crisis are responsible for most of the morbidity and mortality in SCD (Bunn, 1997). VOC are caused by sickled red blood cells which obstruct capillaries and restrict blood flow to an organ, resulting in ischemia, pain and organ damage. VOC is the most frequent crisis that results in tissue ischemia, organ failure, pain and, occasionally, death (Embry et al., 1994; Hebbel, 1997). Because of its narrow vessels and function in clearing defective red blood cells, the spleen is frequently affected.

The sites of acute painful crisis vary for each patient. Pain occurs commonly in the extremities, thorax, abdomen, and back. Pain tends to recur at the same site for a particular person. For each person, the quality of the pain is usually similar from one crisis to another (Ballas & Delengowski, 1993). Bone is also a common target of vaso-occlusive damage, especially when the bone is weight-bearing. The pain experienced by sickle-cell
patients is partly due to bone ischemia. Such damage may result in avascular necrosis (especially of the femur) and bone deterioration (Hernigou et al., 1993).

2.1.7 Epidemiology and prevalence of SCD

The disease afflicts about 100 million people worldwide with high prevalence in Africa (Ohene-Frempong & Nkrumah, 1994) and also people of Mediterranean, Caribbean, Middle Eastern and Asian origin (Npat, 2002). According to the World Health Organization (WHO), 5.2% of the world’s population and over 7% of pregnant women carry an abnormal haemoglobin gene (Modell & Darlinson, 2008). About 300,000 children are born annually with SCD and most affected children develop multiple strokes and die before they are two years of age (Serjeant, 1997); making it a major public health problem on a global scale (Gladwin et al., 2004). In the United States, SCD is the most common genetic disorder with more than 80,000 people affected, the majority of whom are African Americans (NHLBI-NIH, 2009).

In Africa, SCD is reported to be associated with a very high rate of 50% to 90% childhood mortality (Diallo, 2008). It also affects an estimated 1 to 2% of newborns in Africa annually with sickle cell gene most common in areas where malaria is endemic (Meremikwu, 2008). SCD is most prevalent in sub-Saharan Africa and Nigeria has the highest incidence of SCD worldwide with approximately 45,000 to 90,000 babies born each year with SCD in the country (SCDAA, 2007).

Reports from Ghanaian studies indicate a carrier rate in the population of 30% whereas 2% of Ghanaian newborns have sickle cell disease (Ohene-Frempong et al., 2008).
2.2 PATHOPHYSIOLOGY OF SICKLE CELL DISEASE

2.2.1 Haemoglobin

Haemoglobin (Hb) was one of the first proteins to be sequenced (Christopher et al., 2013). Human haemoglobin is a heterotetramer molecule that consists of two pairs of identical globin polypeptide subunits, each encoded by a different family of genes (Okpala, 2004). Globin polypeptides are synthesized from α-like globin genes (ζ, α1, and α2) located on chromosome 16 and β-like globin genes (ε, Gγ, Aγ, δ, and β) located on chromosome 11 (Okpala, 2004). Nascent globin chains rapidly incorporate haem, which stabilizes their native folding into Hb subunits composed of seven or eight alpha helices named, which fold together into a globular structure (Christopher et al., 2013). The major function of Hb is to transport oxygen from the lungs to peripheral tissues and carbon dioxide from the tissues to the lungs.

The normal red blood cell contains three types of haemoglobin. During fetal life, the predominant type of haemoglobin is haemoglobin F (Hb F [α2γ2]) (Okpala, 2004). After birth, Hb F is gradually replaced by haemoglobin A (Hb A [α2β2]) which approximately accounts for 95% of the circulating haemoglobin in normal adult individuals (Okpala, 2004). Haemoglobin A2 (Hb A2 [α2δ2]) is a minor adult-type haemoglobin that accounts for less than 2.5% of the circulating haemoglobin in normal individuals in adult life (Bank, 2006).

Mutations that alter any step in globin processing, including subunit folding, haem interaction, dimerization or tetramerization can destabilize Hb.
2.2.2 Haemoglobinopathies

Haemoglobinopathy is defined as a genetic defect that results in abnormal structure of one of the globin chains of the haemoglobin molecule. These variants alter haemoglobin structure and biochemical properties with physiological effects ranging from insignificant to severe (Christopher et al., 2013). So far, more than 800 haemoglobin variants have been diagnosed (Huisman et al., 1998). Haemoglobinopathies represent a major portion of all inherited diseases worldwide affecting an estimated 7% of the world’s population (Weatherall & Clegg 2001; Kohne, 2011). By convention, these variants are named after the geographic origin of the affected individual (Christopher et al., 2013).

Variations from normal haemoglobin can be classified into three major categories: structural variants, thalassemias and hereditary persistence of fetal haemoglobin (HPFH) (Okpala, 2004). Structural haemoglobin variants result from mutations of which majority are point mutations that give rise to the formation of globin with an abnormal structure. More than 1000 naturally occurring human haemoglobin variants with single amino acid substitutions throughout the molecule have been discovered, mainly through their clinical and/or laboratory manifestations (Christopher et al., 2013). The most common and medically important Hb variants include HbS (Serjeant & Rodgers, 2012; Williams & Weatherall, 2012), HbC (Lettre, 2012; Serjeant & Rodgers, 2012; Williams & Weatherall, 2012), Hb E (Vichinsky, 2007) and Hb D-Punjab (Tyagi et al., 2000).

Thalassemia result from mutations that cause a defect in the synthesis of one or more globin chains disturbing the ratio of alpha to non-alpha chains (Okpala, 2004). Beta-thalassemia results from deletional, frameshift and point mutations and can be divided into two types: beta zero and beta plus thalassemias. Beta zero occur when no beta chains are
produced and beta plus when there is reduced chain production. Alpha thalassemia is primarily caused by gene deletions. Again the nomenclature of alpha plus and alpha zero is used. This relates to the number of genes which are non-functional and therefore to the amount of alpha chains produced and thus clinical severity (Okpala, 2004).

HPFH refers to a benign group of conditions in which the synthesis of fetal haemoglobin remains raised throughout life (Okpala, 2004)

2.2.3 Haemoglobin S
Sickle haemoglobin (HbS) is a structural variant of normal adult haemoglobin (HbA) caused by a mutation in the β-globin gene (Chakravorty & Williams, 2015). The sickle cell gene (βS) is as a result of a point mutation (GAG---GTG) in the sixth codon of the gene (Odievre et al, 2011). Due to this mutation, the sixth amino acid in the beta chain of HbS is valine, instead of glutamic acid as found in the normal adult HbA (Galkin & Vekilov, 2004). This alteration is the basis of all the problems that occur in people with sickle cell disease.

DNA polymorphisms or haplotypes around the βS-globin locus exist which are named after the region of first description. Four major sickle haplotypes of the βS-globin exist in Africa: “Senegal” (West Africa), “Benin” (Central West Africa), “Bantu” or “CAR” (Central African Republic) and “Cameroon” (Dorn-Beineke & Frietsch, 2002). An “Asian haplotype” occurs particularly in East Saudi Arabia and Central India. Haplotypes differ in their severity, frequency and pattern of clinical symptoms; however, the factors influencing the differences between them are still not clarified (Dorn-Beineke & Frietsch, 2002).
The sickle cell gene is prevalent in Africans, Saudi Arabians, Iranians, Asiatic Indians, Malaysians, Italians, Turks, Greeks and Afro-Americans. In Central and Western Africa, the homozygous state (HbSS) occurs in 3% of the population and the heterozygous form “sickle cell trait” (HbAS) occurs in 25% of the population. Among black Americans in North, Middle and South America, the incidence of HbSS is 0.15% and 8% of HbAS. In Jamaica, 10% of the population have sickle cell trait. In recent data, 30,000 patients with sickle cell trait and 800 to 900 homozygous sickle cell patients are reported to be living in Germany (Dorn-Beineke & Frietsch, 2002).

Due to the Hb S mutation and its pathogenesis, heterozygotes are nearly resistant to malaria since infected red blood cells are more susceptible to sickling and intracellular parasites are lysed by sickling. The frequency of the sickle gene in a population therefore indicates the historical incidence of malaria (Dorn-Beineke & Frietsch, 2002).

2.2.4 HbS polymerisation and sickling

The primary pathogenic event of sickle cell disease is the polymerization of the mutant haemoglobin (HbS) within the red blood cells. It occurs under deoxygenated conditions in venous circulation (Galkin & Vekilov, 2004). HbS Polymerization accounts for the fragility of the red blood cells and thus for the haemolytic anaemia (Odievre et al., 2011).

Ordinarily, the haemoglobin molecules exist as single, isolated units in the red blood cell, whether they have oxygen bound or not. Sickle haemoglobin molecules also exist as isolated units in the RBC when they have oxygen bound. However, deoxygenation which follows the passage of RBCs in microcirculation endows HbS molecules with a new
property: thus the capacity to polymerize. This is due to the replacement of the hydrophilic glutamic acid at position 6 in the β-globin chain by the hydrophobic valine in HbS. Upon deoxygenation, valine residue establishes hydrophobic interactions with other hydrophobic residues on the β-globin chain of another deoxy-HbS molecule (Odievre et al., 2011). HbS molecules tend to stick together and form long chains of polymers. HbS polymers then group together and stiffen, distorting the cell and inducing the characteristic SS-RBC shape change, classically in the shape of a sickle (Odievre et al., 2011). HbS polymerisation cause damages to the sickle erythrocyte membrane, among such changes is their propensity to adhere (Browne et al., 1998; Kaul et al., 2009).

The process of polymerization is described by a double-nucleation mechanism (Eaton & Hofrichter, 1990). The first step in polymerization is homogeneous nucleation, in which single fibers are randomly generated in the bulk of a supersaturated solution followed by their growth. As the fibers grow, they serve as substrates for the nucleation of new fibers (Briehl, 1995). The second step is the branching of new fibers on top of pre-existing ones. This step is referred to as heterogenous nucleation (Eaton & Hofrichter, 1990).

An important feature of Hb polymerization process and its subsequent sickling is the “delay time”. The delay time is the time the process of polymerization needs to be primed. Studies of the kinetics of HbS polymerization have shown a latency phase preceding the formation of the polymer strands and their explosive growth inside the erythrocyte referred to as “delay time”. In basal conditions, the delay time is longer than the time of passage of RBCs in the microcirculation. Hence, most red blood cells escape sickling during the capillary transit due to the fact that the delay time for HbS polymerization is generally longer than the capillary transit times of red blood cells (<1 sec) (Kaul et al.,
2009). This explains why most sickle patients, who have sufficient HbS in each red blood cell to sickle are not sicker than they are.

The delay time plays a crucial role in the pathophysiology of sickle cell disease. If the delay time for HbS polymerization is shorter than the capillary transit time, sickling will occur in the capillaries with likely vaso-occlusion. If the delay time is prolonged so that it exceeds the capillary transit time, polymerization and sickling would occur in the venules, with no vaso-occlusion (Brugnara, 1995). Hence any factor that shortens the delay time accelerates polymerization and will promote microcirculatory vaso-occlusion.

It has been reported that the delay time is inversely proportional to the 30\textsuperscript{th} to 50\textsuperscript{th} power of HbS concentration (Cao & Ferrone, 1996). In other words, sickle red blood cells with high haemoglobin concentration will be most susceptible to accelerated HbS polymerization. Hence, small changes in HbS concentration markedly affect Hb polymerization and cell sickling (Brugnara, 1995). Dehydration of red blood cells containing sickle haemoglobin will therefore contribute to the pathophysiology of sickling because of the high dependence of polymerization on the concentration of HbS.
2.3 SICKLE CELL DEHYDRATION

Maintenance of water and solute homeostasis is critical to the survival of the red blood cell. Several pathways mediate water and solute homeostasis in normal red blood cells where cell volume is primarily controlled through regulation of intracellular monovalent cation content (Brugnara, 1993). RBCs swell when their inward sodium leak exceeds the potassium leak out and shrink when the potassium leak out exceeds the inward sodium leak. In view of this, a fine homeostatic mechanism which is mediated by several pathways ensures a balance in ion fluxes, ion content regulation and the hydration state (cell volume) of the cell which is critical for its survival (Lew & Bookchin, 2005). In the normal RBC membrane, this homeostatic mechanism is remarkably constant for most of their 120-day life span. Therefore, inherited or acquired disorders that perturb this homeostasis will jeopardize the erythrocyte, leading to its destruction (Rinehart et al., 2010).

One of the distinguishing characteristics of sickle erythrocytes is the presence of cell dehydration. The fine homeostatic balance described above for normal RBCs is markedly altered in sickle cells, whose ion fluxes, ion content regulation and hydration state becomes disrupted (Lew & Bookchin, 2005). The formation of the deoxy-HbS polymer fibers in the red blood cell triggers a whole series of changes of the red blood cell membrane. Some ion channels are activated and it results in the dysregulation of cation homeostasis leading to an outward loss of potassium accompanied by chloride ions and osmotically obliged water, hence cellular dehydration occurs. Cellular dehydration in turn, by increasing the intracellular HbS concentration contributes to the pathophysiology of sickling because of the high dependence of polymerization on the concentration of HbS (Odievre et al., 2011).
Dehydration of human RBCs result from the activation of one or more of three transporters expressed in their plasma membranes: 1) the Ca\(^{2+}\) -sensitive, K\(^{+}\)-selective channel (Gardos channel) (Vandrope et al., 1998; Hoffman et al., 2003), also expressed in many other cell types (Lew & Bookchin, 2005) 2) a K\(^{+}\)-Cl\(^{-}\) Cotransporter (KCC) (Lauf et al., 1992; Franco et al., 1995; Gillen et al., 1996) and 3) the Na\(^{+}\) pump (Skou & Esmann, 1992). These three transporters differ considerably in their dehydrating modalities, potencies, and distributions among RBCs. For the purpose of this study, K\(^{+}\)-Cl\(^{-}\) cotransporter will be considered.

### 2.4 K\(^{+}\)-Cl\(^{-}\) Cotransporter (KCC)

K\(^{+}\)-Cl\(^{-}\) cotransporter (KCC) is a gradient-driven transport system. KCC is expressed in a large variety of cell types and is involved in cell volume regulation (Bize & Dunham, 1995). KCC mediates an electroneutral symport of K\(^{+}\) and Cl\(^{-}\) ions across the plasma membrane that is mostly outwardly directed accompanied with water and was originally described in RBC (Rust et al., 2007). KCC activity in RBC is a major determinant of RBC volume and density and its activity is increased in SCD (Rust et al., 2007) contributing to RBC dehydration and the formation of sickle cells (Joiner et al., 2007).

Human RBC K\(^{+}\)-Cl\(^{-}\) cotransporter activity is influenced by the oxygenation state of haemoglobin and is regulated by numerous activators and inhibitors. For example, cell swelling, thiol oxidation, cellular magnesium depletion, slight acidification (by lowering pH to 7), protein kinase inhibitors and free radicals are activators, whereas cell shrinkage,
most bivalent cations, marked acidification (below pH 6.5), alkalinization (above pH 7.4), polyamines and protein phosphatase inhibitors are inhibitory (Adragna et al., 2004).

The functional state of the transporter is however positively regulated by phosphorylation and dephosphorylation of serine/threonine and tyrosine residues of the polypeptide chain of the cotransporter (Gillen et al., 1996) by the enzymes protein phosphatases-1 (PP1) and/or protein phosphatases-2A (PP2A) (Starke & Jennings, 1993; Bize et al., 2003; Lew & Bookchin, 2005).

Several studies with inhibitors of protein phosphatases and kinases reported the capability of Src family kinases (a subtype of non-receptor protein-tyrosine kinases) to downregulate PP1 activity. Src family kinases phosphorylate and inhibit PP1 activity. De Francheschi et al., (1997), reported that genetic deficiency of Fgr and Hck; two tyrosine kinases of the Src family, results in elevated K-Cl cotransport activity in mouse erythrocytes, indicating a role for these kinases in the regulation of RBC K-Cl cotransport activity (De Francheschi et al., 1997). Also further studies by De Francheschi et al., reported that Fgr phosphorylates PP1α (an isoform of PP1) in vitro (De Francheschi et al., 2001) as well as demonstrating that K⁺-Cl⁻ cotransport is activated in erythrocytes lacking Hck and Fgr kinases (De Francheschi et al., 2001). These findings suggest that Src family kinases act as upstream negative regulators of PP1 and that their increased activity downregulates K-Cl cotransport activity.
2.5 MAGNESIUM

2.5.1 Chemical characteristics

Magnesium (Mg) is the eighth most common element in the crust of the earth (Jahnen-Dechent & Ketteler, 2012). It is a Group 2 (alkaline earth) element within the periodic table and has a relative atomic mass of 24.305Da, a specific gravity at 20°C of 1.738, a melting point of 650°C and a boiling point of 1090°C (Jahnen-Dechent & Ketteler, 2012). Mg is readily available to organisms and it is the fourth most abundant cation in vertebrates (Maguire et al., 2002). It is essential, especially within cells, being the second most common intracellular cation being vital for numerous physiological functions (Swaminathan, 2003).

2.5.2 Distribution in the human body

The total magnesium content in the average 70 kg adult body with 20% (w/w) fat is reported to be approximately 1000 to 1120 mmol/L (Jahnen-Dechent & Ketteler, 2012) or approximately 24 g (Fox et al., 2001; De Baaij et al., 2015). Only 1 to 2 % of the total magnesium is stored in extracellular compartment, 67% is stored in the bone whiles 31% is intracellular (Akhtar et al., 2011). Intracellular magnesium concentrations range from 5 to 20 mmol/L. In the cell, 1 to 5% of magnesium is ionized, the remainder is bound to proteins, negatively charged molecules and adenosine triphosphate (ATP) (Rude, 1996). Mg ions enter cells by a passive mechanism due to the electrochemical gradient that results from the electronegative charge of the cells interior and by facilitated diffusion (Pearson et al., 1998).
Extracellular magnesium accounts for approximately 1% of total body magnesium (Rude, 1996) which is primarily found in serum (Touyz, 2004). Serum magnesium can be categorized into three fractions. About one-third is bound to proteins, mainly albumin; the remaining two-thirds are ultrafiltrable, being approximately 92% free/ionized and 8% complexed to citrate, phosphate, and other compounds (Pearson et al., 1998). Ionized magnesium has the greatest biological activity (Touyz, 2004). Serum concentrations of magnesium range between 0.7 and 1.1 mM in healthy people (De Baaij et al., 2015).

### 2.5.3 Mg balance and consumption in the human body

Mg balance in the body depends on the collaborative actions of the intestine, responsible for Mg uptake from food, the bone, which stores Mg in its hydroxy-apatite form, and the kidneys, regulating urinary Mg excretion (De Baaij et al., 2015). The daily intake of magnesium is approximately 20 to 30 meq/day (240 to 370 mg/day) corresponding to 30 to 40% of the total amount taken in diet which is absorbed in the distal small bowel. The reabsorption of magnesium by the kidneys is very efficient; 25% of filtered Mg is reabsorbed in the proximal convulated tubule, where as 50 to 60% is reabsorbed in the thick ascending limb of the loop of Henle. Magnesium balance is therefore achieved through intestinal absorption and renal excretion (Jahnen-Dechent & Ketteler, 2012).

Magnesium stored in bones is not completely bioavailable during magnesium deprivation (Maguire & Cowan, 2002) hence humans need to consume magnesium regularly to prevent magnesium deficiency. The recommended daily allowance for magnesium varies; it is difficult to define accurately what the exact optimal intake should be. Values of 300 mg are usually reported with adjusted dosages for age, sex and nutritional status (Jahnen-
Dechent & Ketteler, 2012). The United States Food and Nutrition Board have recommended a daily Mg intake of 420 mg for men and 320 mg for women (De Baaij et al., 2015). Drinking water accounts for approximately 10% of daily magnesium intake. Foods such as nuts, seeds, green vegetables, legumes, fruit, meat, fish and whole grains are rich sources of magnesium in diet. Processed foods have low magnesium content and as such dietary intake of magnesium in the western world is reported to be decreasing owing to the increased consumption of processed food (Ford & Mokdad, 2003). Dairy products have been found to also contain low magnesium concentrations (Jahnen-Dechent & Ketteler, 2012).

2.5.4 Physiological role of Mg

Over the last decade, clinical interest in Mg has been growing and considered as a treatment for several major diseases. Mg is critical to the function of basically every organ in the human body. Mg is primarily found within the cell (Jahnen-Dechent & Ketteler, 2012) where it acts as a counter ion for the energy-rich ATP. Magnesium is a cofactor in more than 300 enzymatic reactions (Swaminathan, 2003). Mg critically stabilizes enzymes, including many ATP generating reactions (Jahnen-Dechent & Ketteler, 2012). ATP is required for glucose utilization, synthesis of fat, proteins, nucleic acids and coenzymes, muscle contraction and many other processes therefore interference with magnesium metabolism also influences these functions (Jahnen-Dechent & Ketteler, 2012). ATP metabolism, muscle contraction and relaxation, normal neurological function and release of neurotransmitters, heart and lung function are all magnesium dependent. Mg also contributes to the regulation of bone formation (Cunningham et al., 2012). Again, Mg further modulates insulin signal transduction and cell proliferation, important for cell
adhesion and transmembrane transport including transport of potassium and calcium ions. It also maintains the conformation of nucleic acids and is essential for the structural function of proteins and mitochondria (Jahnen-Dechent & Ketteler, 2012).

2.5.4.1 Role of Mg in CNS

In neuronal function, Mg levels in the CNS contribute to the hyperexcitability of excitatory neuronal pathways (Paoletti et al., 2013), the regulation of oxidative stress and the release of neuropeptides such as calcitonin gene-related peptide (CGRP). CGRP is secreted from sensory neurons and has a vasodilatory effect (Bigal, 2013). Also, Mg enhances the activity of neuronal nitric oxide synthases (nNOS) and nitric oxide (NO) produced has multiple functions in the brain including vasodilation, regulation of gene transcription, channel activity and neurotransmitter release (Steinert et al, 2010).

2.5.4.2 Role of Mg in Lungs

Mg is known to have vasodilatory effect (Teregawa et al., 2002). In the lungs, like many vasodilators, Mg also has a bronchodilating effect (Hirota et al., 1999) although the mechanisms underlying Mg-induced bronchodilation remain to be elucidated. Moreover, Mg also inhibits the release of acetylcholine (Ach) and histamine, both known to induce bronchoconstriction (Rowe & Camargo, 2008).

2.5.4.3 Role of Mg in Heart

In heart function, Mg influences myocardial metabolism, Ca homeostasis and cardiac output in three ways: It regulates the activity of ion channels in the cardiac cells, thereby affecting the electrical properties of the myocardium (Mubagwa et al., 2007). It also regulates myocardial contractility by influencing the intracellular Ca mobility (De Baaij et
Mg also has an anti-inflammatory and vasodilatory effect (De Baaij et al., 2015).

2.5.4.4 Role of Mg in skeletal muscles

In skeletal muscles, Mg mainly exerts its effects as a calcium antagonist on calcium permeable channels and calcium binding proteins. In the resting state, Mg\(^{2+}\) is present in concentrations 10,000 times higher than Ca\(^{2+}\) in muscle cells (Konishi, 1998) and it occupies all Ca\(^{2+}\) binding sites. Mg is only displaced from the Ca binding site after Ca\(^{2+}\) is released from the sarcoplasmic reticulum. However, in Mg\(^{2+}\)-deficient conditions, not as much Ca\(^{2+}\) is required to displace Mg\(^{2+}\), resulting in hypercontractibility, which presents as muscle cramps and spasms in the clinic.

2.5.5 Magnesium imbalances in the body

2.5.5.1 Hypomagnesaemia

Hypomagnesaemia is generally defined as serum Mg levels below 0.7 mM and it is considered severe below 0.4mM (Godron et al., 2012). Hypomagnesaemic patients usually suffer from nonspecific symptoms such as depression, tiredness, muscle spasms and muscle weakness (Godron et al., 2012). Hypomagnesaemia has prevalence of 7% to 11% observed in patients hospitalized in wards (Swaminathan, 2003) and 20% to 60% in intensive care units (Jahnen-Dechent & Ketteler, 2012). Hypomagnesaemia is also reported to be associated with increased mortality in intensive care patients. Secondary to hypomagnesaemia, disturbances in other electrolytes such as K\(^{+}\) and PO\(_4\)\(^{3-}\) in 40% of cases and to a lesser extent Na\(^{+}\) and Ca\(^{+}\) are often detected (Soave et al., 2009; Godron et al., 2012).
Hypomagnesaemia is associated with a wide range of diseases (De Baaij et al., 2015). It is reported to contribute to the development of diabetes mellitus type 2 by increasing insulin resistance (Hubbard, 1997). Mg deficiency has also been reported in children with bronchitis (Bednarek et al., 2003; Sabbagh et al., 2006). Chronic magnesium deficiency has also been linked to atherosclerosis, myocardial infarction, hypertension, malignant tumors, kidney stones, alteration in blood lipids, premenstrual syndrome and psychiatric disorders (Swaminathan, 2003). Furthermore, hypomagnesaemia is associated with a wide range of neurological diseases such as migraine, depression and epilepsy (Paoletti et al., 2013). Hypomagnesaemia is also reported to be associated with several disorders of SCD (Nduka et al., 1995; Oladipo et al., 2005).

Furthermore, it is reported that Mg deficiency exacerbates anaemia in SCD patients and in mouse models of sickle cell disease and β-thalassemia (Olivieri et al., 1994; De Francheschi et al., 1997). Magnesium also plays a crucial role in the modulation of endothelial inflammation, which may be beneficial in SCD (Makis et al., 2000; Maier et al., 2004; Britain & Parise, 2007). Magnesium has also been shown to slow clotting time, which might reduce vascular blockage and improve blood flow in SCD (Assaley et al., 1998).

Clinically, magnesium deficiency is commonly associated with chronic inadequate intake of Mg, diarrhea, vomiting and laxative intake abuse (De Baaij et al., 2015). The use of certain drugs which are associated with renal magnesium wasting such as diuretics, cisplatin, aminoglycosides, digoxin, furosemide, amphotericin B, cyclosporine A (Jahnen-Dechent & Ketteler, 2012) leads to hypomagnesaemia. Several endocrine disorders such as parathyroid disease, hyperaldosteronism is also reported to be linked with
hypomagnesaemia (Agus, 1999). Chronic alcoholism also results in hypomagnesaemia (Pearson et al., 1998; De Baaij et al., 2015). Hypomagnesaemia also occur perioperatively and is commonly found in patients undergoing cardiothoracic or major abdominal surgery or thyroidectomies (Susanne et al., 2011).

Although hypomagnesaemia is associated with a wide range of diseases, magnesium status in patients is frequently undetected. Mg is described as the most under diagnosed electrolyte abnormality in current medical practice (Limaye et al., 2011) and the “forgotten” cation in human health (De Baaij et al., 2015). Measurement of serum magnesium concentration in 1,000 samples received for electrolyte determination showed that only 10% of the hypomagnesaemic patients had magnesium requested. Therefore routine assessment of magnesium status is advised in all acutely ill patients especially in those with conditions, diseases or treatment that may predispose to magnesium deficiency (Swaminathan, 2003).

2.5.5.2 Hypermagnesaemia

Hypermagnesaemia is generally defined as serum Mg levels above 1.1 mM. Hypermagnesaemia is less frequent than hypomagnesaemia. It may be clinically observed in patients suffering from nausea, vomiting, lethargy, headaches, and/or flushing (De Baaij et al., 2015).

Moderate hypermagnesaemia is frequent in patients with chronic renal insufficiency, because of rhabdomyolysis, due to the release of magnesium from disintegrating muscle after excessive use of antacids or laxatives containing magnesium salts (Fung et al., 1995). Severe hypermagnesaemia is most often observed during the therapeutic administration of
magnesium sulfate in patients with chronic renal insufficiency or during treatment of
eclampsia (Morisaki et al., 2000).

2.5.6 Role of magnesium in sickle RBC dehydration

A distinguishing feature of SCD is the presence of dense, dehydrated erythrocytes. Under
hypoxic conditions, red blood cell dehydration results in increased sickle cell HbS
haemoglobin concentration, accelerated polymerization, sickling in microcirculation and
possible vaso-oclusion. (Brugnara, 1995; Odievre et al., 2011). Dehydration of sickle red
blood cells contributes greatly to the pathophysiology of sickling and its possible vaso-
occlusion in microcirculation. Magnesium levels have been shown to regulate red blood
cell hydration (De Franceschi et al., 2000).

In SCD, HbS polymerization is associated with abnormal changes in RBC membrane
permeability during deoxygenation with subsequent efflux of potassium accompanied with
water through the activation of two important pathways: the Gardos channel (Vandorpe,
1998); and the K-Cl co-transporter (Brugnara, 1995). The activity of the K-Cl co-transport
is affected by RBC magnesium content. (De Franceschi et al., 1997).

The functional/active state of KCC is positively regulated by protein phosphatases PP1
and/or PP2A by a dephosphorylation reaction (Bize et al., 2003; Lew & Bookchin, 2005).
It is reported that Src family kinases act as upstream negative regulators of PP1 and that
their increased activity downregulates K-Cl cotransport activity (De Franceschi et al.,
2001). However, activity of Src family kinases, like other tyrosine kinases is dependent on
the binding of two Mg $^{2+}$ ions (Hubbard, 1997).
Considering the relationship between kinase function and magnesium, experimental trials on mouse strains bred to select for high and low magnesium levels in erythrocytes was developed to study the role of high and low Mg levels on activity of KCC transporter implicated in cell volume regulation. It was reported that K-Cl cotransporter activity was elevated in erythrocytes of mouse strains bred to select for low magnesium levels, in parallel with decreased erythrocyte enzymatic activity of Src family kinases. In addition, the expression and activity of PP1 varied inversely with cell Mg content and was higher in erythrocytes of mouse strains bred to select for low magnesium levels (De Francheschi et al., 2001).

These findings suggest that inhibition of K-Cl cotransport activity and erythrocyte dehydration by elevated Mg is due to reduced PP1α activity resulting from enhanced modulation of Src family kinase activities. Mg therefore regulates K-Cl cotransport in vivo by enhancing Src family kinase activities and the consequent inhibition of PP1α activity then decreases K-Cl cotransport activity (De Francheschi et al., 2001). By increasing red blood cell magnesium, the abnormal activation of K-Cl cotransporter is blocked, thereby inhibiting efflux of potassium and loss of water from the cell.

Again, K-Cl cotransporter is stimulated by oxidative stress and other activators following dephosphorylation by protein phosphatase (Olivieri et al., 1994; Brugnara, 1994). A work on the role of high and low magnesium levels on erythrocyte susceptibility to oxidative stress reported a moderately higher susceptibility of mouse strains bred to select low magnesium levels in erythrocytes to oxidative stress (De Francheschi et al., 2001). This oxidative vulnerability due to low Mg levels in RBC’s may influence the K-Cl cotransporter activity (De Francheschi et al., 2001).
Currently, magnesium salts (MgSO$_4$) are used in clinical practice to reduce erythrocyte dehydration in SCD (De Francheschi et al., 1997). It has also been observed that oral magnesium supplements such as magnesium pidolate, have significantly improved red cell hydration by reducing the number of dense sickle erythrocytes, absolute reticulocyte count and immature reticulocytes, whiles erythrocyte magnesium content were significantly increased (De Franceschi et al., 1997; De Franceschi et al., 2000; Zehtachi et al., 2004). De Franceshi (1996) also reported that dietary Mg supplements improved erythrocyte abnormalities and increased haemoglobin in an “SAD” mouse (a transgenic model of SS disease) (De Franceshi et al, 1996).

### 2.5.7 Role of magnesium in vascular function

Magnesium plays very important roles in regulating vascular homeostasis and a mediator of endothelial functions. It directly influences vascular tone, baseline tension and vascular responsiveness to vasoconstrictor agents both through endothelium independent and endothelium dependent pathways (Cunningham et al., 2012). As a result of its role in vascular functions, a significant inverse correlation exist between serum magnesium levels and incidence of cardiovascular diseases (Bo & Pisu, 2008; Cunha et al., 2012). Hypertensive patients are also reported to generally exhibit reduced intracellular concentrations of magnesium compared to non-hypertensive subjects (Touyz et al., 1992; Cunha et al., 2012).

Extracellular magnesium content have been shown to modify the production and release of nitric oxide (NO) (Cunha et al., 2012), a potent vasodilator which plays very important roles in the vascular system by regulating vascular homeostasis. Some studies have
confirmed increased production of NO by magnesium, promoting endothelium-dependent vasodilation (Northcott & Watts, 2004; Yang et al., 2000). Furthermore, MgSO₄, infused intravenously in increasing doses showed an increased peripheral and cerebral flows and decreased cerebral and systemic vascular resistances (Torregrosa et al., 1994; Nakaigawa et al., 1997). Pearson et al (1998) also demonstrated that hypomagnesaemia selectively impaired the release of NO from coronary endothelium. Since NO is a potent endogenous vasodilator and an inhibitor of platelet aggregation and adhesion, magnesium deficiency will therefore promote Mg-induced vasoconstriction (Pearson et al., 1998).

Interestingly, microvessels are more dependent on the Mg-induced vasodilatation on endothelial cell release of NO implicating Mg as an important mediator in reducing sickle cell vaso-oclusion in microcirculation (Yang et al., 2000).

Again, in smooth muscle cells of blood vessels, Mg influence vascular tone by acting as a calcium channel blocker which affects cytoplasmic calcium ion concentrations and its availability at critical sites (Barbagallo & Dominguez, 2007). Antagonistic action of Mg is mainly by its blockage of voltage-gated Ca channels in smooth muscle cells membrane (Fleckenstein-Grun et al., 1997) and also inhibiting Ca release from intracellular stores of smooth muscle cells (Torregrosa et al., 1994). A reduction in cytoplasmic calcium inhibits intracellular signal transduction pathways in smooth muscle cells that lead to vasoconstriction. Relaxation of blood vessels therefore occurs in decreased Ca concentrations in smooth muscle cells (Altura & Altura, 1995). Elevation of extracellular Mg is reported to result in decreased Ca levels in smooth muscle cells (Altura & Altura, 1995) which may be the primary mechanism by which extracellular Mg-induced endothelium independent relaxation is brought about (Yang et al., 2000).
Magnesium deficiency in vasculature is also reported to promote oxidative stress notably in endothelial cells (Wiles et al., 1997), resulting in increased reactive oxygen species (ROS) and cytotoxicity (Zhou et al., 1999). In this condition of low Mg-induced oxidative stress, the endothelium develops a state of permanent inflammation, recruiting monocytes and triggering the proliferation and migration of vascular smooth muscle cells eventually resulting in atherosclerosis, vascular calcifications, or thrombosis (De Baaij et al., 2015). Elevation of extracellular magnesium levels also potentiates the dilatory action of some endogenous (adenosine, potassium and some prostaglandins) and exogenous (isoproterenol and nitroprusside) vasodilators which result in mild reduction of systolic pressure (Shechter et al., 2000). Although many of these mechanisms explaining Mg role in maintaining vascular homeostasis remain controversial and in some cases speculative, the beneficial effects related to the consequences of Mg supplementation are apparent.

2.6 NITRIC OXIDE

2.6.1 Synthesis

Nitric oxide (NO) is a soluble gas with a half-life of approximately 6-30 seconds, synthesized from the amino acid L-arginine by a family of enzymes called the nitric oxide synthases (NOS) (Thomas et al., 2013). There are three isoforms of NOS; NOS found in endothelial cells (eNOS) and neurons (nNOS) which are both constitutively expressed enzymes and whose activities are stimulated by increases in intracellular calcium. Immune functions for NO are mediated by a calcium-independent inducible NOS (iNOS) isoform (Wood et al., 2006). The eNOS, derived from vascular endothelium, is the most dominant form of these isoforms. Other sources such as cardiac myocytes, blood platelets and the brain are also important in nitric oxide production (Thomas et al., 2013).
2.6.2 Mechanism of action of NO

Nitric oxide is released in the endothelium by increases in intracellular calcium in response to various receptor-mediated stimuli (including acetylcholine, ATP, ADP, histamine, bradykinin substance P and thrombin), nonreceptor-mediated stimuli (such as calcium influx) and mechanical stimuli (Howard et al., 1995) which increases calcium influx as well as efflux from intracellular stores in the endothelial cell. Increased intracellular Ca in endothelium leads to the activation of endothelial nitric oxide synthase with its subsequent production of NO from L-arginine. NO released from the endothelium activates soluble guanylyl cyclase in smooth muscle after binding to its heme group, resulting in increased intracellular cyclic GMP (Mack & Kato, 2006). Cyclic GMP activates cGMP-dependent kinases that decrease intracellular calcium concentration in smooth muscle, producing relaxation and vasodilation. This effect increases regional blood flow (Mack & Kato, 2006). In addition, NO induces a coordinated programme of cellular events that promote blood flow, primarily by suppressing platelet aggregation, expression of cell adhesion molecules on endothelial cells, and secretion of procoagulant proteins (Reiter et al., 2002; Rother et al., 2005; Voetsch et al., 2004).

2.6.3 Role of NO in SCD

Nitric oxide produced by the endothelial cells has been considered as a very potent vasodilator and has blood flow regulatory effect. Enhanced vasodilatory effect of NO in SCD may decrease the severity of vaso-occlusive crisis (Lopez et al., 1996). Also decreased bioavailability of NO result in VOC partly due to an increase in the production of the vasoconstrictor peptide endothelin-1.

Recent investigations have revealed that the pathophysiology of SCD is not limited to the HbS polymerization but includes the sickle RBCs, white blood cells and platelets
adhesiveness to the vascular endothelium in SCD patients (Manwani & Paul, 2013). Thus, event of vaso-occlusion in sickle cell disease start with the adhesion of sickle erythrocytes, leukocytes and platelets to adhesion molecule on activated endothelial cells predominantly in the post capillary venules. Increased adherence of sickle red blood cells to endothelium is implicated as an initiating event of vaso-occlusion in sickle cell disease. NO is reported to inhibit sickle RBC, platelets and leukocytes to endothelium and decreases expression of vascular cell adhesion molecule-1 (VCAM-1), an endothelial adhesion site implicated in sickle RBC/endothelial adherence (Space et al., 2000).
CHAPTER THREE
METHODOLOGY

3.1 Study design

This study was a cross sectional study which compared SCD patients from the Centre for clinical Genetics and apparently healthy HbAA individuals from Accra Area Blood Centre for National blood donation, the biggest tertiary sickle cell clinic and blood donation centers in Ghana respectively.

3.2 Study sites

The study was conducted at the Korle-Bu Teaching Hospital. The Korle-Bu Teaching Hospital situated in the nation’s capital, Accra, Ghana, is the leading tertiary hospital and the major referral centre in the country. The hospital was founded in 1923 as the Gold Coast Hospital. The then governor, Gordon Guggisberg, laid the foundation for Korle-Bu Hospital in 1921, and it was opened on 9th October, 1923. The hospital has expanded in phases and now has over 2000 beds with three centres of excellence, the National Cardiothoracic Centre, the National Plastic and Reconstructive Surgery and the Radiotherapy Centre. The hospital also serves as the teaching hospital for the University of Ghana, College of Health Sciences.

The Centre for clinical Genetics and Accra Area Blood Centre for National blood donation are the biggest tertiary sickle cell clinic and blood donation centres respectively in the country. Most of the sickle cell patients within the nation’s capital Accra receive their treatment at the sickle cell clinic. The population within Accra is composed of different social and ethnic groups as well as geographically distinct areas. Therefore, the demographics of the study participant who were enrolled in this study were not limited to a specific social group.
3.3 Study participants

A total of 371 subjects were recruited for the study. This was made up of 80 HbAA individuals (59 males, 21 females), 97 HbSS SCD patients in steady state (50 males, 47 females), 75 HbSS SCD patients in VOC (31 males, 44 females), 56 HbSC SCD patients in steady state (22 males, 34 females) and 63 HbSC SCD patients in VOC (26 males, 37 females). Study subjects recruitment was done with the approval of the College of Health Sciences Ethical and Protocol Review Committee of the University of Ghana and the participant’s consent.

3.4 Inclusion criteria

SCD patients with HbSS or HbSC genotype written in their medical records at the clinic, aged between 15 and 65 years in steady state or VOC.

Steady state was clinically defined as a patient who has been well and has not been in crisis for at least 2 weeks and is going about his activities. Vaso-occlusive crisis was also clinically defined as pain in the bones, muscles and joints not attributable to any other cause and requiring parenteral analgesia and admitted in the Centre for some hours. Vaso-occlusive crisis was diagnosed by the Physician on duty attending to the patients.

3.5 Exclusion criteria

SCD patients with other genotypes other than HbSS and HbSC written in their medical records were excluded. Voluntary blood donors who tested positive for sickling test were excluded. Study subjects below 15 and above 65 years of age were also excluded.
3.6 Sample size determination

The minimum sample size was determined by the use of software from: http://sampsize.sourceforge.net/iface/s3. With the following values:

Minimum Odds Ratio to detect = 2
Percentage exposed among controls = 40%

Power = 90
Number of controls per case = 1
Alpha risk = 5%

1:1 matched study design

The minimum sample size for SCD cases was 180. The SCD categories of interest were HbSS and HbSC. Based on the preliminary data available from the Center for Clinical Genetics, the incident ratio of HbSS: HbSC is 3:1 respectively. Therefore the minimum samples sizes for HbSS:HbSC was 120:60 patients according to the ratio. This was age matched with 80 HbAA individuals as comparison group. The same group of HbAA individuals was compared with all the SCD categories.
3.7 Methods

3.7.1 Sampling Technique

A random sampling method was used to select for SCD patients and HbAA individuals.

3.7.2 Participants demographics

The nationality, tribe, sex, age and occupation of all the participants was obtained through the administration of questionnaires.

3.7.3 Collection of blood samples, preparation and storage

The method as described in the Standard Operating Procedure for performing venepuncture in the National Blood Bank (Korle-bu) was used. Rubber tourniquet was tied to the biceps about 8cm above the elbow joint for less than one minute and the site to be punctured was cleansed with methylated spirit. Then 5mls of blood was drawn from the brachial vein with a 19G hypodermic needle fixed on 5ml syringe. All aseptic conditions were adhered to. Aliquots of 2.5ml of the blood sample from each subject was immediately transferred into coded Na EDTA and plain tubes. The blood in the Na EDTA tube was mixed in the bottles to prevent clotting by gently inverting the tubes four times manually. The blood sample in the plain tube was spun at a speed of 2500 rpm for 10mins to separate serum from cells. Serum obtained was stored at -20°C.
3.7.4 Measurement of haematological profile

Full blood count analysis of samples in Na EDTA tubes was done on the same day of collection using lab system multiskan MS (manufactured by Amisham Bioscience LTD, UK), a three-part auto analyzer able to run 19 parameters per sample including haemoglobin concentration, packed cell volume, red blood cell concentration, mean corpuscular haemoglobin, mean cell volume, mean corpuscular haemoglobin concentration, white blood cells and platelet parameters.

3.7.5 Estimation of serum magnesium, calcium, potassium and sodium levels

Determination of serum magnesium and calcium was done using a Flame Atomic Absorption Spectrometer (Variant 240FS manufactured by VARIAN Australia Pty Ltd). Serum sodium and potassium were also analyzed using Sherwood Flame Photometer (Model 420 by Sherwood Scientific Ltd). All electrolyte measurements were done at the Chemistry Unit of the Ghana Atomic Energy Commission.

3.7.5.1 Reagents

- 30% of concentrated Hydrogen Peroxide (H₂O₂)
- 65-67% of concentrate Nitric Acid (HNO₃)
- Acetylene
- 100mg/L Lithium solution

3.7.5.2 Glass ware and apparatus

- 50ml measuring cylinder
- 100ml class ‘A’ beaker
- Test tubes
- Fume chamber
- Clean film
- Hot plate
- A 3ml dropper
- Wash bottle

3.7.5.3 Procedure

Two (2) grams of serum sample was weighed into a 100ml class A beaker. Twelve (12ml) of conc. HNO₃ was added to the weighed sample. This was done in the fume chamber. The beaker was then covered with a clean film and placed on a hot plate for a hot plate acid digestion. The sample was digested for 3 hours at a temperature of 45°C. After the acid digestion, the sample was transferred into a 50ml measuring cylinder. The sample was topped up to the 30ml mark with distilled water. The whole content was then transferred into a test tube for the electrolyte analysis. Reference standards and blanks were also digested under the same conditions as the samples. These served as internal positive controls. Reference standards used were from Fluka Analytical, Sigma-Aldrich Chemie GmbH, a product of Switzerland.

The digestate was used directly for magnesium and calcium assessment using the Atomic Absorption Spectrometer in an acetylene-air flame. For potassium analysis, 5ml of the digestate was transferred into a 10ml measuring cylinder and 2ml of lithium solution was added to the 5ml digestate and measurement was taken. For sodium analysis, a 1:5 water dilution of the digested sample was transferred in a 10ml measuring cylinder. Two (2ml) of lithium solution was added to the 1:5 diluted digestate in the measuring cylinder and measurement was taken. Sodium and potassium were measured using the Sherwood Flame Photometer (Model 420). All electrolyte determinations were done in duplicates.
3.7.5.4 Quality control and assurance techniques used

The blanks were used to check contamination during sample preparation. Measurements were done in duplicates to check the reproducibility of the method used. The standards used were to check the efficiency of the equipment used.

Table 3.1: Atomic absorption spectrophotometer working conditions

<table>
<thead>
<tr>
<th>ELEMENT</th>
<th>WAVELENGTH /nm</th>
<th>LAMPCURRENT /Ma</th>
<th>SLIT WIDTH /nm</th>
<th>FUEL</th>
<th>SUPPORT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg</td>
<td>285.5</td>
<td>4</td>
<td>0.5</td>
<td>Acetylene</td>
<td>Air</td>
</tr>
<tr>
<td>Ca</td>
<td>422.7</td>
<td>10</td>
<td>0.5</td>
<td>Acetylene</td>
<td>Nitrous oxide</td>
</tr>
</tbody>
</table>

VARIAN. Publication No 85-10009-00 Revised March 1989.

3.7.6 Measurement of nitric oxide

Nitric oxide levels were assessed in the plasma samples using the Griess Reagent system (Promega, Madison, USA). The assay relied on a diazotization reaction that was originally described by Griess (1879). The Griess Reagent System is based on the chemical reaction which uses sulfanilamide and N-1-naphtylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. This system detects Nitrite in a variety of biological and experimental liquid matrices such as plasma, serum, urine and tissue culture medium.
3.7.6.1 Preparation of a Nitrite Standard Reference Curve

A Nitrite Standard reference curve was prepared for each assay in order to accurately quantify nitrite levels in experimental samples. A volume of 1ml of a 100μM nitrite solution was prepared by diluting the provided 0.1M Nitrite Standard (0.1M sodium nitite in water) 1:1,000 in distilled water. Three columns (16 wells) in the 96-well plate were designated and labeled appropriately for the Nitrite Standard reference curve. A volume of 50μl of the distilled water was dispensed into the wells in rows with a micropipette. 100μl of the 100μM nitrite solution was added to the remaining 3 wells in row. Serial twofold dilutions (50μl/well) were done in triplicate to generate the Nitrite Standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56μM) by plotting the average absorbance value of each concentration of the Nitrite Standard on the Y-axis and the nitrite concentration on the X-axis (Figure 3.1).

![Figure 3.1: Nitrite Standard Reference Curve](University of Ghana http://ugspace.ug.edu.gh)
From this graph the formula $Y=0.0066x + 0.053$ was derived and was used to calculate the nitrite concentration. Values on the Y-axis are the average absorbances (490nm) and values on the X-axis are the nitrite concentration ($\mu$M).

### 3.7.6.2 Determination of Nitrite concentration as an index for NO in experimental samples.

Sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) solutions were allowed to equilibrate to room temperature for about 20 minutes. Aliquots of 50$\mu$l each of the experimental plasma samples were added to the remaining wells in triplicate and labeled with a marker. Using a multi channel pipette, 50$\mu$l of the Sulfanilamide solution was dispensed to all experimental samples and the wells containing the dilution series for the Nitrite Standard reference curve. The plate was then incubated for 10 minutes at room temperature, protected from light by keeping in a dark box. After incubation, 50$\mu$l of the NED Solution was dispensed to all the wells using a multichannel pipette. The plate was then incubated again for 10 minutes at room temperature, protected from light in the dark box. A purple/magenta color started to form immediately. The absorbance was then measured at 490nm using a micro plate reader manufactured by Machine Biotek Instruments, USA (Model EL808) after the incubation period.

The average absorbance value of the triplicates of each sample was determined. The concentration of nitrite as an index for NO in the experimental samples was determined by comparison to the nitrite standard reference curve.
3.7 Statistical analysis

Data collected was entered into SPSS software student version 20.0 and used for analysis. All values were expressed as mean (M) ± standard deviation (SD) [M±SD]. The differences between two means were analyzed statistically using the Student $t$-test for unpaired data. Analysis of variance (ANOVA) was used to compare the difference between more than two means of groups of subjects. Pearson’s correlation coefficient ($r$) was used to find the relationship between magnesium and NO levels in subjects. Statistical significance was defined as $p < 0.05$.

3.8 Dissemination of results

Findings of this study was submitted to the Graduate School, University of Ghana and the Department of Physiology under School of Biomedical and Allied Health Sciences, University of Ghana. Presentations will be made at local and international seminars/conferences about the outcome of the study. The final work will be published in a reputable peer review journal.
CHAPTER FOUR

RESULTS

4.1 General characteristics

A total of 371 subjects were sampled. This was made up of 80 HbAA individuals (59 males, 21 females) with mean age of 33.70 ± 11.77 years, 97 HbSS SCD patients in steady state (50 males, 47 females) with mean age of 26.02 ± 10.19 years, 75 HbSS SCD patients in VOC 31 males, 44 females with mean age of 26.30 ± 10.41 years, 56 HbSC SCD patients in steady state (22 males, 34 females) with mean age of 33.16 ± 14.29 years and 63 HbSC SCD patients in VOC (26 males, 37 females) with mean age of 32.71 ± 13.39 years.

4.2 Haematological parameters among male HbAA individuals and SCD patients in steady states and VOC.

The Haematological profiles in males such as haemoglobin (Hb), Haematocrit (HCT), red blood cells (RBC), mean corpuscular haemoglobin concentration (MCHC), Red cell distribution width (RDW), mean platelet volume (MPV), platelets count (Plt), plateletcrit (Pct) and white blood cells (WBC) were statistically significant across board (Table 4.1)
Table 4.1: A comparison of the mean haematological parameters among male HbAA subjects and sickle cell disease patients in steady state and VOC

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HbAA (n=59)</th>
<th>HbSS steady state (n=50)</th>
<th>HbSC steady state (n=22)</th>
<th>HbSSVOC (n=31)</th>
<th>HbSCVOC (n=26)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>15.38± 3.68</td>
<td>8.532± 1.610</td>
<td>12.51± 1.33</td>
<td>9.24± 1.67</td>
<td>12.10± 1.63</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>42.20± 6.51</td>
<td>25.82± 5.15</td>
<td>39.83± 4.63</td>
<td>28.37± 6.01</td>
<td>38.34± 4.37</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RBC (10⁶/mm³)</td>
<td>5.14 ± 0.82</td>
<td>3.06±0.71</td>
<td>4.82± 0.49</td>
<td>3.50± 0.89</td>
<td>4.45± 0.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MCV (µm³)</td>
<td>82.62± 6.09</td>
<td>84.97± 8.47</td>
<td>82.90± 5.15</td>
<td>82.59± 9.45</td>
<td>84.42± 6.20</td>
<td>0.185</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>27.44± 2.67</td>
<td>28.20± 3.51</td>
<td>26.02± 1.83</td>
<td>27.50± 6.95</td>
<td>27.200±2.04</td>
<td>0.274</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>33.18±1.36</td>
<td>33.12±1.46</td>
<td>30.02±1.831</td>
<td>33.03±4.92</td>
<td>32.24±0.58</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>33.71±13.251</td>
<td>18.56±2.54</td>
<td>14.97±1.35</td>
<td>18.56±2.89</td>
<td>15.43±1.16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MPV (µm³)</td>
<td>9.090± 1.383</td>
<td>7.466± 0.686</td>
<td>8.32 ± 1.01</td>
<td>7.652± 0.814</td>
<td>7.875±0.766</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PDW (%)</td>
<td>13.761±1.919</td>
<td>13.915±2.258</td>
<td>14.23±2.23</td>
<td>13.194±3.006</td>
<td>13.42±2.22</td>
<td>0.0900</td>
</tr>
<tr>
<td>PLT(10³/mm³)</td>
<td>224.22±61.17</td>
<td>466.33±121.39</td>
<td>289.64±18.31</td>
<td>439.3±158.77</td>
<td>238.558±109.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PCT (%)</td>
<td>0.18 ± 0.07</td>
<td>0.35 ±0.10</td>
<td>0.23±0.08</td>
<td>0.34±0.12</td>
<td>0.19±0.07</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>WBC(10³/mm³)</td>
<td>5.309±1.107</td>
<td>12.18±3.56</td>
<td>6.87± 1.77</td>
<td>16.24 ±7.37</td>
<td>9.78±2.09</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Mean ± SD. Hb, haemoglobin; HCT, Haematocrit; RBC, red blood cell count; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW, Red cell distribution width; MPV, mean platelet volume; PLT, platelets count; PCT, plateletcrit; PDW, platelets distribution width ; WBC, white blood cells. *signifcant at p≤ 0.05
Pair wise analysis: Student’s t-test analyses of group difference were as follows:

Group difference that were not significant were not included.

I: Hb

a. HbAA x HbSS steady state  p-value = <0.001
b. HbAA x HbSS VOC  p-value = <0.001
c. HbSS steady state x HbSC steady state  p-value = 0.0079
d. HbSS VOC x HbSC steady state  p-value = 0.0418

For the Pair wise analysis: Student’s t-test analyses of group difference for HCT, RBC, MCHC, RDW, PLT, PCT and WBC refer to Appendix III.

4.3 Haematological parameters among female HbAA individuals and SCD patients in steady states and VOC.

The Haematological profiles in females such as haemoglobin (Hb), Haematocrit (HCT), red blood cells (RBC), mean corpuscular haemoglobin concentration (MCHC), Red cell distribution width (RDW), mean platelet volume (MPV), platelets count (Plt), plateletcrit (Pct) and white blood cells (WBC) were statistically significant across board, p>0.05. (Table 4.2)
## Table 4.2: A comparison of the mean haematological parameters among female HbAA subjects and sickle cell disease patients in steady state and VOC

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HbAA (n=21)</th>
<th>HbSS steady state (n=47)</th>
<th>HbSC steady state (n=34)</th>
<th>HbSSVOC (n=44)</th>
<th>HbSCVOC (n=37)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dL)</td>
<td>13.19± 3.32</td>
<td>8.308 ±1.570</td>
<td>10.74± 1.73</td>
<td>8.42 ±1.68</td>
<td>11.26 ±1.50</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>40.91± 10.94</td>
<td>24.84± 5.18</td>
<td>33.02± 7.08</td>
<td>25.58 ±5.74</td>
<td>35.209 ±4.66</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>RBC (10⁶/mm³)</td>
<td>4.97 ± 1.26</td>
<td>2.89±0.77</td>
<td>4.04 ±0.94</td>
<td>3.05 ±0.87</td>
<td>4.28 ±0.56</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>MCV (µm³)</td>
<td>82.62± 5.29</td>
<td>87.44± 8.83</td>
<td>81.96± 8.53</td>
<td>85.50 ±9.45</td>
<td>82.50 ±7.61</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>26.68± 2.05</td>
<td>29.42± 3.60</td>
<td>26.61±2.26</td>
<td>28.40 ±4.02</td>
<td>26.427 ±2.87</td>
<td>0.5410</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>32.29± 0.90</td>
<td>33.59± 1.44</td>
<td>31.86± 1.15</td>
<td>33.11 ±1.69</td>
<td>31.96 ±1.03</td>
<td>0.4588</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>18.587±13.251</td>
<td>17.82± 2.68</td>
<td>15.01±2.58</td>
<td>17.39 ±2.20</td>
<td>14.86 ±1.16</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>MPV (µm³)</td>
<td>8.153± 1.082</td>
<td>7.316± 0.697</td>
<td>8.23±1.01</td>
<td>7.624 ±0.704</td>
<td>7.936 ±0.781</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>PDW (%)</td>
<td>14.909± 1.970</td>
<td>14.101± 1.956</td>
<td>12.66±2.89</td>
<td>13.529 ±2.738</td>
<td>12.79 ±2.22</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>PLT(10⁹/mm³)</td>
<td>250.82± 80.77</td>
<td>461.95± 160.55</td>
<td>322.87±20.70</td>
<td>459.09±193.64</td>
<td>319.00±114.88</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>PCT (%)</td>
<td>0.19 ±0.06</td>
<td>0.33 ±0.11</td>
<td>0.26 ±0.09</td>
<td>0.35 ±0.14</td>
<td>0.25 ±0.08</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>WBC(10³/mm³)</td>
<td>5.793 ± 1.282</td>
<td>10.69±3.11</td>
<td>9.32 ±2.56</td>
<td>13.01 ±4.83</td>
<td>8.25 ±1.52</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

Mean ± SD. Hb, haemoglobin; HCT, Haematocrit; RBC, red blood cell count; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW, Red cell distribution width; MPV, mean platelet volume; PLT, platelets count; PCT, plateletcrit; PDW, platelets distribution width; WBC, white blood cells. Significant at p≤ 0.05.
Pair wise analysis: Student’s t-test analyses of group difference were as follows:

Group difference that were not significant were not included

I: Hb

a. HbSC \text{VOC} \times \text{HbSS steady state} \quad p\text{-value}= <0.001
b. Control \times \text{HbSS steady state} \quad p\text{-value}= <0.001
c. \text{HbSC steady state} \times \text{HbSS \text{VOC}} \quad p\text{-value}= <0.001
d. \text{HbSC \text{VOC}} \times \text{HbSS \text{VOC}} \quad p\text{-value}= <0.001
e. \text{HbAA} \times \text{HbSS \text{VOC}} \quad p\text{-value}= <0.001
f. \text{HbAA} \times \text{HbSC steady state} \quad p\text{-value}= <0.001
g. \text{HbAA} \times \text{HbSC \text{VOC}} \quad p\text{-value}= <0.001

For the Pair wise analysis: Student’s t-test analyses of group difference for HCT, RBC, RDW, MCV, PLT, PCT and WBC refer to Appendix IV.
4.4 A comparison of serum magnesium levels in HbAA individuals and SCD patients in steady states and VOC.

In a cross sectional study of serum magnesium levels in SCD patients in different disease states and HbAA individuals, the mean serum magnesium levels was generally higher in the HbAA individuals (2.46± 0.64) mEq/L than in all the SCD patient categories thus; HbSS SCD patients in steady state (1.928±0.60) mEq/L, HbSS SCD patients in VOC (1.920 ± 0.57) mEq/L, HbSC SCD patients in steady state (1.848 ± 0.35) mEq/L, HbSC SCD patients in VOC is (1.845 ± 0.10) mEq/L (figure 4.1). The differences between these means were significant (p < 0.05).

Figure 4.1: A comparison of serum magnesium levels in sickle cell disease categories and HbAA individual. HbAA; apparently healthy individuals, HbSS; SS genotype SCD patients in steady state, HbSC; SC genotype SCD patients in steady state, HbSSvoc; SS genotype SCD patients in crisis, HbSCvoc; SC genotype SCD patients in crisis.
4.5. A comparison of serum sodium levels in HbAA individuals and SCD patients in steady states and VOC.

In a multiple comparison of the five mean serum sodium levels by one-way ANOVA, the mean serum levels in SCD was generally lower as compared to HbAA individuals, p < 0.001.

The post hoc analysis revealed that there was a high significant difference in serum sodium level between both HbSS (178.63±32.24)mEq/L and HbSC SCD (210.61±69.20) mEq/L patients in steady state were significantly higher than HbSC (115.93±11.90) mEq/L and HbSS (113.07± 94.61)mEq/L in VOC (p<0.001) (figure 4.2).

Figure 4.2: A comparison of serum sodium levels in sickle cell disease categories and HbAA individuals. HbAA; apparently healthy individuals, HbSS; SS genotype SCD patients in steady state, HbSC; SC genotype SCD patients in steady state, HbSSvoc; SS genotype SCD patients in crisis, HbSCvoc; SC genotype SCD patients in crisis.
4.6 A comparison of serum potassium levels in HbAA individuals and SCD patients in steady states and VOC.

Figure 4.3 shows the multiple comparison of the five mean serum potassium levels by one-way ANOVA and the mean serum potassium level in the HbAA individuals was generally higher compared to all the SCD categories (p < 0.001).

The post hoc analysis revealed that there was a high significant difference in serum potassium level between both HbSS (11.29 ±2.24) mEq/L and HbSC SCD (11.20 ±1.20) mEq/L patients in steady state were significantly higher than HbSC (9.004 ±0.71) mEq/L and HbSS (8.018 ± 0.61)mEq/L in VOC (p<0.001). (figure 4.3).

![Figure 4.3: A comparison of serum potassium levels in sickle cell disease categories and HbAA individuals. HbAA; apparently healthy individuals, HbSS; SS genotype SCD patients in steady state, HbSC; SC genotype SCD patients in steady state, HbSSvoc; SS genotype SCD patients in crisis, HbSCvoc; SC genotype SCD patients in crisis.](image-url)
4.7 A comparison of serum calcium levels in HbAA individuals and SCD patients in steady states and VOC.

Comparing the serum calcium levels in SCD patients categories and HbAA individuals, the mean serum calcium levels was generally higher in the HbAA controls (11.383 ± 1.60) mEq/L than the SCD patients; HbSS SCD patients in steady state (9.98 ± 0.60)mEq/L, HbSS SCD patients in VOC (10.010 ± 0.57)mEq/L, HbSC SCD patients in steady state (9.848 ± 0.54)mEq/L, HbSC SCD patients in VOC is (9.855 ± 0.11)mEq/L. The differences between these means were significant (p < 0.05). Refer to figure 4.4 below.

Figure 4.4: A comparison of serum calcium levels in sickle cell disease categories and HbAA individuals. HbAA; apparently healthy individuals, HbSS; SS genotype SCD patients in steady state, HbSC; SC genotype SCD patients in steady state, HbSCvoc; SS genotype SCD patients in crisis, HbSCvoc; SC genotype SCD patients in crisis.
4.8 A comparison of plasma NO levels in HbAA individuals and SCD patients in steady states and VOC.

Figure 4.5 below shows a comparison between plasma NO levels in HbSS and HbSC SCD patients in different disease states and controls. The HbAA controls recorded significantly higher NO level (49.01± 6.72)µM compared to all the other subject categories of SCD (p<0.001). HbSS in VOC NO was significantly lower (10.56± 2.33)µM (p<0.001). (figure 4.5).

![Figure 4.5: A comparison of plasma NO levels among different sickle cell disease genotypes and disease states with HbAA individuals. HbAA; apparently healthy individuals, HbSS; SS genotype SCD patients in steady state, HbSC; SC genotype SCD patients in steady state, HbSSvoc; SS genotype SCD patients in crisis, HbSCvoc; SC genotype SCD patients in crisis](http://ugspace.ug.edu.gh)
4.9 Association between serum Mg and NO in HbAA individuals

There was a very weak negative association between serum Mg and plasma NO in HbAA individuals \((r = -1.020)\). (figure 4.6).

![Figure 4.6: Correlation between serum Mg and plasma NO in HbAA individuals](image)

\[
y = -1.020 \times + 22.091 \\
R^2 = 0.001
\]
4.10 Association between serum Mg and plasma NO in HbSC SCD patients in steady states.

There is a very weak positive association between serum Mg and plasma NO in HbSC SCD patients in steady state ($r = 1.112$). This means that higher serum Mg levels are associated with higher levels of NO in HbSC SCD patients in steady state. The coefficient of determination ($r^2 = 0.004$). (figure 4.7).

![Graph showing correlation between serum Mg and plasma NO](image)

**Figure 4.7: Correlation between serum Mg and plasma NO in HbSC SCD patients in steady state**
4.11 Association between serum Mg and plasma NO in HbSS SCD patients in steady states.

There is a very weak positive association between serum Mg and plasma NO in HbSS SCD patients in steady state \( (r = 0.660) \). This means that higher serum Mg levels are associated with higher levels of NO in HbSS SCD patients in steady state. The coefficient of determination \( (r^2 = 0.00) \). (figure 4.8).

![Figure 4.8: Correlation between serum Mg and plasma NO in HbSS SCD patients in steady state](image-url)
4.12 Association between serum Mg and plasma NO in HbSC SCD patients in VOC

There is a very weak negative association between serum Mg and plasma NO in HbSC SCD patients in VOC ($r = 0.905$). This means that higher serum Mg levels are associated with lower levels of NO in HbSC SCD patients in VOC. The coefficient of determination ($r^2 = 0.002$). (figure 4.9).

Figure 4.9: Correlation between serum Mg and plasma NO in HbSC SCD patients in VOC

$$y = -0.905x + 31.084$$

$$R^2 = 0.002$$
4.13 Association between serum Mg and plasma NO in HbSS SCD patients in VOC

There is a very weak positive association between serum Mg and plasma NO in HbSS SCD patients in VOC ($r = -1.823$). This means that higher serum Mg levels are associated with lower levels of NO in HbSS SCD patients in VOC. The coefficient of determination ($r^2 = 0.003$). (figure 4.10).

![Graph showing correlation between serum Mg and plasma NO](image)

**Figure 4.10: Correlation between serum Mg and plasma NO in HbSS SCD patients in VOC**
CHAPTER FIVE
DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Sickle cell disease (SCD) is a hereditary red blood cell disorder found in black populations across the world and a major genetic disease in Ghana with a wide variety of clinical manifestations. A variety of electrolyte abnormalities in SCD including Mg is well reported (Neely et al., 1969; Karayalin et al., 1981; Nduke & Ekeke, 1986; El-Hazmi et al., 1989; Mohammed et al., 1993) and the possible roles that these play in the pathophysiology of the disease have been proposed (Nduka et al., 1995). This study therefore examined serum magnesium and other major electrolytes including sodium, potassium and calcium levels in HbSS and HbSC SCD patients in steady state and during VOC compared with age matched healthy HbAA individuals.

The results obtained for the haematological parameters for male and female study subjects showed that, the red cell indices such as Hb, MCV, MCH and MCHC were generally lower in HbSS SCD patients than in HbAA individuals while the white blood cell and platelet counts were higher in HbSS SCD patients than in HbAA individual. Furthermore, these red cell indices were higher in males than females in both SCD and HbAA subjects. This result was consistent with the results reported in previous studies (Omoti, 2005; Ehsan et al., 2010, Okocha et al., 2011; Antwi-Boasiako et al., 2015). These results were to be expected considering the degree of chronic haemolysis and higher risk of infections in SCD patients. Blood viscosity increases with increase in PCV, therefore most SCD patients have adapted to low red cell indices in view of the fact that raising the PCV to over 30% could increase the viscosity of the blood that could precipitate in VOC (Antwi-Boasiako et al., 2015). Due to redistribution of the WBC between the marginal and
circulating pools, pain, nausea, vomiting and anxiety have been reported to cause leucocytosis in the absence of infection (Antwi-Boasiako et al., 2015). Epidemiologic data also indicate that higher WBC count is associated with poor prognosis (Powers, 1991; Okpala, 2004) while reduced neutrophil count is associated with good prognosis. Elevated WBC count in SCD patients may also be due to autosplenectomy resulting from recurrent splenic vessels occlusion, which makes patients more susceptible to overwhelming infections like *Streptococcus pneumonia* and *Haemophilus influenza*. Increased erythropoesis due to androgens in males, and iron loss or blood loss in females during menstruation may be responsible for higher levels of the red cell indices in males compared to females (Kato et al., 2009).

The mean serum Mg levels were significantly lower in SCD patients than in HbAA individuals, however, the levels between steady state and VOC in SCD patients were not significantly different. There was also no significant difference among the SCD genotypes. These outcomes of the study is consistent with earlier studies which reported low plasma Mg levels in SCD patients (Olukoga et al., 1990; Zehtachi et al., 2004; Emokpai & Musa, 2015). However, Akenami et al., (1999) reported elevated serum magnesium levels in SCD patients as compared to controls. Another study also reported no significant difference of circulating Mg levels in SCD patients as compared to controls (Oladipo et al., 2005). These contrasting observations to this study may be as a result of ethnic difference since serum Mg levels has been associated with ethnicity and appears to show racial differences, with African Americans having lower values than Caucasians (Resnick et al., 1997; Fox et al., 1999). At the level of the SCD RBC membrane, Mg$^{2+}$ is a tonic inhibitor of K$^+$–Cl$^-$ cotransporter that is integral in controlling RBC cell volume (De Francheschi et al., 2001; Laires et al., 2004). The low intracellular magnesium level,
common in SCD patients, promotes K⁺–Cl⁻ cotransport, thus resulting in increased K⁺ efflux and cellular dehydration (De Franceschi et al., 1997; De Franceschi et al., 2000). Restoring intracellular magnesium from low levels in SCD to normal levels inhibits K⁺–Cl⁻ cotransport, decreasing K⁺ efflux and ameliorating RBC hydration. The Mg deficiency in SCD may be an intrinsic part of the disease as a consequence of renal losses (Olukoga et al., 1993), genetic errors in Mg metabolism (Henrotte et al., 1990) or possibly diminished dietary consumption (Ford, 1997; Altura et al., 2002) by the patients.

In this study, total magnesium was measured as an indicator of the magnesium status in the subjects. A number of studies have suggested that only the ionic magnesium pool accurately reflects magnesium status, since ionic magnesium is the biologically active form of magnesium. Other reports have also suggested that the total serum magnesium pool does not accurately reflect changes in the ionic Mg pool (Maj-Zurawska, 1997; Barrera et al., 2000; Sinert et al., 2007; Johansson & Whiss, 2007). However, other studies have proven that total and ionic magnesium serum concentrations are strongly correlated in chronic diseases such as SCD, and that either gives an accurate assessment of magnesium status but not in critically ill patients which reported a poor correlation (Saha et al., 1996; Saha et al., 1998; Mikhail & Ehsanipoor, 1999; Longstreet & Vink, 2009). Therefore, serum total magnesium determination as used in this study is sufficient for the assessment of magnesium status in chronic diseases including SCD while serum ionic magnesium determination may be a better indicator of magnesium status in acute disease states (Longstreet & Vink, 2009).

Sodium and potassium mean levels were lower in SCD patients in steady state as compared to apparently healthy HbAA individuals, with a further decrease during VOC as represented in figures 4.2 and 4.3. The results is in agreement with other studies in which lower levels of these electrolyte have been reported in patients with SCD in steady state
and in crisis (Tosteston et al., 1952; Passow, 1969; Clark et al., 1981; Brugnara, 1993; Brugnara, 2000; Ibe et al., 2009; Agoreyo & Nwanze 2010). In SCD, sickle erythrocytes exhibit abnormal changes in membrane permeability and that deoxygenated sickle erythrocytes have increased Na and K fluxes, with net cellular Na gain and K loss (Bookchin & Lew, 1981). There is also increased and continued obligatory losses of body fluids and electrolytes which rapidly result in dehydration (Sergeant, 1992). Dehydration causes Na, K and other electrolyte imbalance in the body. Consequently, more water and electrolytes like sodium and potassium are lost from the body. Again, in SCD there is suppression of appetite and patient may not drink. There is also high skin loss of electrolytes coupled with the obligatory urinary losses from inability to concentrate urine which all lead to Na, K and other electrolyte imbalance (Ibe et al., 2009).

The low levels of Na and K reported in this study is therefore suggested to be due to reduced fluid intake, accelerated influx and efflux of sodium and potassium, increased insensible loss and high incidence of hyposthenuria in the SCD patients (Vikas et al., 2012). Also, the excessive accumulation of intracellular calcium in SCD activates the Gardos channel which then expels potassium into the renal tubules (Vikas et al., 2012). Again, in normal erythrocyte and oxygenated sickle RBC the passive leak rates for Na and K are approximately 1-2 mEq/1013 RBC/hr however when sickle RBC are deoxygenated, the passive rate of cation transfer increases five or six fold (Passow, 1969; Vikas et al., 2012). The mechanism by which sickling alter normal Na and K permeability is not clearly defined although it is most likely due to physical distortion of normal cation permeability barriers (Vikas et al., 2012).

The mean serum Ca level in SCD patients was significantly lower as compared with HbAA individuals. Calcium levels between steady state and VOC in SCD patients were not significantly different as well as among the SCD genotypes. This observation is in
agreement with previous studies who also reported a similar trend (Al-Dabbagh et al., 1989; Mohammed et al., 1993; Nduka et al., 1995; van der Dijs et al., 1997). The insignificant difference in calcium levels in patients in steady state and those in VOC as well as Ca levels between HbSS and HbSC patients in this study was also reported by Nduka et al., (1995) Red blood cell membrane abnormalities with increased cation permeability and a deoxygenation-induced cation conductance which mediates Ca$^{2+}$ entry into cells (van der Dijs et al., 1997) and subsequent accumulation of calcium in the RBC of patients with sickle cell has been reported (Oladipo et al., 2005). These factors are likely contributors to the reduced serum calcium levels in SCD patients as shown in this study. Again, some other reasons given for hypocalcemia in SCD include an increased Ca$^{2+}$-Mg$^{2+}$ ATPase, reduced calcium absorption from the intestinal tract and impaired vitamin D synthesis (Oladipo et al, 2005).

The study revealed that NO levels were significantly high in HbAA individuals than in SCD patients in steady state and further significantly higher than SCD in VOC both the HbSS and HbSC patients. This result was consistent with earlier studies which reported low levels of NO in SCD patients compared to controls (Lopez et al., 1996; Lopez et al., 2000; Morris et al., 2000; Antwi-Boasiako et al., 2015). SCD patients are reported to suffer from decreased NO reserves (Gladwin et al., 2004). Also, blood plasma levels of L-arginine are lower depressed in patients with SCD, particularly during VOC and these levels vary inversely with pain symptoms (Morris et al., 2000). Again, NO-dependent blood flow is reported to be impaired in SCD patients (Belhassen et al., 2001; Gladwin et al., 2004). The low levels of NO in SCD may also result from conditions that interfere with NO bioavailability (Marin & Rodriguez-Martinez, 1997) such as elevated levels of cell-free hemoglobin in plasma resulting from haemolysis in SCD. The cell free
hemoglobin in plasma rapidly destroys or mops up NO (Kaul & Hebbel, 2000; Reiter & Gladwin, 2003) thereby limiting NO bioavailability (Reiter et al., 2002). Therefore, the impaired bioavailability of NO in SCD as reported in this study which is consistent with previous studies appear to be due to decreased plasma L-arginine a precursor for NO biosynthesis and consumption of NO by cell-free plasma haemoglobin and by reactive oxygen species. During VOC, it was expected that NO will play its compensatory role in maintaining vascular function by rising to reverse the occlusion. Although lack of NO may not be the actual cause of VOC, its presence will ameliorate the crisis if not prevent it entirely. Therefore, failure of the NO compensatory system to operate during VOC due to lack of NO bioavailability aggravates the crisis. Again, during the process of VOC the balance of local vasoconstrictors and vasodilators like NO is altered in favor of vasoconstriction. This may account for the vasoconstriction that enhances VOC caused by the sickled erythrocyte (Antwi-Boasiako et al., 2015).

This study reports a positive correlation between serum magnesium and plasma NO levels in the HbSS and HbSC of the SCD patients. However, there was a negative correlation between magnesium and NO levels in both HbSS and HbSC in VOC. There are no data in literature to compare these results with. Extracellular magnesium content have been shown to modify the production and release of nitric oxide (NO) (Cunha et al., 2012). Yang et al., 2000 reported that serum magnesium produces a concentration-dependent NO release from the intact rat aortic rings. Other studies have also confirmed increased production of NO by magnesium, promoting endothelium-dependent vasodilation (Northcott & Watts, 2004; Yang et al., 2000). Furthermore, MgSO₄, infused intravenously in increasing doses showed an increased peripheral and cerebral flows and decreased cerebral and systemic vascular resistances (Torregrosa et al., 1994; Nakaigawa et al., 1997). Pearson et al (1998) also demonstrated that hypomagnesaemia selectively impaired the release of NO from
coronary endothelium. Although many of these mechanisms explaining Mg role in NO production and in maintaining vascular homeostasis remain controversial, the beneficial effects related to the consequences of Mg supplementation are apparent in several studies (Kawano, et al., 1998; Shechter et al., 2000; Haenni et al., 2002; Guerrero-Romero et al., 2004; Almoznino-Sarafian et al., 2009; Guerrero-Romero & Rodriguez-Moran, 2009; Hatzistavri et al., 2009).

5.2 Conclusion

The mean serum Mg and Ca levels were significantly lower in SCD patients regardless of the clinical state as compared with HbAA individuals. Serum Na and K levels were decreased in SCD patients as compared to HbAA individuals and decreased further during VOC. Nitric oxide levels were significantly higher in HbAA than in SCD patients during the steady state regardless of the genotype. Nitric oxide levels were further reduced low during VOC. This study revealed a weak positive association between Mg and NO levels in the steady states in SCD. However, there was a weak negative correlation with SCD patients in VOC and HbAA individuals. Generally electrolytes levels were low in SCD patients as compared to controls. This study confirms electrolyte abnormality in SCD patients.
5.3 Clinical relevance of this study

The clinical relevance of this study could be used to support the previous studies suggesting the use of oral magnesium supplementation as part of the management of SCD. Use of this treatment modality has been shown to prevent erythrocyte dehydration, decrease the frequency of painful crises, and improve RBC indices in SCD patients.

5.4 Limitation

Although, all of this study has suggested the presence of magnesium and other electrolytes deficiency in SCD patients, it did not measure the ionized form of magnesium to confirm that its therapy is beneficial in terms of the biologically active form of Mg.
REFERENCES


De Franceschi, L., Beuzard, Y., Jouault, H., Brugnara, C. Modulation of erythrocyte potassium-chloride cotransport, potassium content and density by dietary magnesium intake in transgenic SAD mouse. Blood., 2738-44.


APPENDICES

Appendix I:
Consent form

You are being asked to volunteer in a research study. This form explains the study. It is important that you understand the study before deciding to be in it. You may ask the persons in charge of the study who are listed on this page questions about the study at any time.

TITLE OF STUDY: SERUM MAGNESIUM LEVELS IN SICKLE CELL DISEASE PATIENTS IN GHANA

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GENERAL INFORMATION:
Sickle cell disease is an inherited blood disorder that affects red blood cells. People with sickle cell disease have red blood cells that contain an abnormal type of hemoglobin. Sickle cell disease is characterized primarily by chronic anaemia and periodic episodes of pain during crisis.
Currently, the prevalence rate of SCD in Ghana is 2% of all births annually.

It has been demonstrated that sickling and subsequent onset of crisis is associated with low levels of magnesium in the sickle RBCs. In Ghana, data linking magnesium levels, the disease presentation and its severity are missing. Data is therefore needed to be collated in that regard, hence the focus of the current study.
WHO CAN BE IN THIS STUDY?
You may participate in this study voluntary if you have sickle cell disease with genotype HbSS and HbSC. This study will involve approximately 371 people of the age group 15 to 65 years older.

VOLUNTEER CONSENT:

Cost of being part of study: There will be no charge from you for the tests involved in this study. It’s absolutely free from your side.

Possible benefits: There are no direct benefits to you from this study. However, your participation may help us develop better management procedures for sickle cell patients in Ghana.

Sample collection: A trained personnel will insert a needle into a vein in one of your arms and draw blood of about two tablespoons from you once for the entire study. This may cause some pain and discomfort at the site of needle insertion.

Possible risk: There are no other risk except the little pain and discomfort during needle insertion.

Information handling: All information will be maintained on confidential basis. Your identity will be protected to the extent permitted by law.

VOLUNTEER AGREEMENT:
I …………………………………………………………………………………………………………… voluntarily agree to participate in this study by donating my blood sample. I certify that the purpose, potential benefits and risk as well my confidentiality have been fully explained/translated to me.

……………………………………… Date ……………………
(Signature/Thumbprint of subject)

………………………………………… Date …………………
(Signature of investigator)
Appendix II:

SCD study subjects questionnaire

Study Date: ______/_____/_____ (dd/mm/yyyy)  Study ID number________
Tel. Number(s)............................................................................

PARTICIPANT INFORMATION

Name: ....................................................................................................................
Age:...................

Surname       First name       Middle name(s)

Sex:    Male [  ] Female [  ]

Tribe:   Ga[  ] Akan [  ] Ewe [ ] Mole-Dagbane [ ] Guan [ ] Ga-Adangbe [ ]

Other (Please specify): .................................................................

Occupation:.................................................................

Place of residence: ..............................................................

Genotype:    [SS]           [SC]

B.CHEMISTRY

1. Estimation of serum levels of electrolytes

2. Estimation of NO

C.HEMATOLOGICAL PROFILE

1. Estimation of WBC, RBC, LYM%, MON%, GRA%, HGB, HCT, MCV, MCH, RDW, PLT, PCT and PDW
Appendix III:

A comparison of the mean haematological parameters among male HbAA subjects and sickle cell disease patients in steady state and VOC (Table 4.1).

Pair wise analysis: Student’s t-test analyses of group difference were as follows:

Group difference that were not significant are not included.

II: HCT

a. HbAA x HbSS steady state  
p-value = <0.001

b. HbAA x HbSS VOC  
p-value = <0.001

c. HbAA x HbSC VOC  
p-value = 0.039

d. HbSC steady state x HbSS steady state  
p-value = <0.001

e. HbSC steady state x HbSS VOC  
p-value = <0.001

f. HbSC VOC x HbSS steady state  
p-value = <0.001

g. HbSC VOC x HbSS VOC  
p-value = <0.001

h. HbSS VOC x HbSS steady state  
p-value = 0.0055

III: MCH

a. HbSS steady state x HbSC steady state  
p-value = 0.011

IV: MCHC

a. HbSC VOC x HbSC steady state  
p-value = <0.001

b. HbSS VOC x HbSC steady state  
p-value = <0.001

c. HbSS steady state x HbSC steady state  
p-value = <0.001

d. HbAA x HbSC steady state  
p-value = <0.001
V: RDW

a. HbSSvoc x HbSC steady state  p-value = 0.0289
b. HbSS steady state x HbSC steady state  p-value = 0.0188
c. HbAA x HbSS steady state  p-value = <0.001
d. HbAA x HbSC\textsubscript{VOC}  p-value = <0.001
e. HbAA x HbSS\textsubscript{VOC}  p-value = <0.001
f. HbAA x HbSS steady state  p-value = <0.001

VI: PLT

a. HbSC steady state x HbAA  p-value = 0.0145
b. HbSS\textsubscript{VOC} x HbAA  p-value = <0.001
c. HbAA x HbSS steady state  p-value = <0.001
d. HbSS steady state x HbAA  p-value = <0.001
e. HbSSvoc x HbSC\textsubscript{VOC}  p-value = <0.001
f. HbSS steady state x HbSSvoc  p-value = <0.001
g. HbSSvoc x HbSC steady state  p-value = <0.001
h. HbSS steady state x HbSC steady state  p-value = <0.001
VII: MPV

a. HbSC steady state x HbSS steady state  p-value = 0.0063
b. HbAA x HbSS steady state  p-value = <0.001
c. HbAA x HbSSvoc  p-value = <0.001
d. HbAA x HbSCvoc  p-value = <0.001
e. HbAA x HbSC steady state  p-value = <0.001

VIII: PCT

a. HbSSvoc x HbSCvoc  p-value = 0.0001
b. HbAA x HbSCvoc  p-value = <0.001
c. HbSS steady state x HbSCvoc  p-value = <0.001
d. HbSS steady state x HbSC steady state  p-value = <0.001

VIX: PDW

a. HbSS steady state x HbSSvoc  p-value = 0.0454
b. HbSC steady state x HbSSvoc  p-value = <0.001
Appendix IV:

A comparison of the mean haematological parameters among female HbAA subjects and sickle cell disease patients in steady state and VOC (Table 4.2)

Pair wise analysis: Student’s t-test analyses of group difference were as follows:

Group difference that were not significant are not included

II: WBC

a. HbSCvoc x HbAA  
   p-value = 0.0141
b. HbSC steady state x HbAA  
   p-value = <0.001
c. HbSS steady state x HbAA  
   p-value = <0.001
d. HbSSvoc x HbAA  
   p-value = <0.001
e. HbSS steady state x HbSCvoc  
   p-value = 0.0101
f. HbSSvoc x HbSC_{VOC}  
   p-value = <0.001
g. HbSS steady state x HbSC steady state  
   p-value = 0.0451
h. HbSS_{VOC} x HbSC steady state  
   p-value = <0.001
i. HbSS_{VOC} x HbSS steady state  
   p-value = <0.001

III: RBC

a. HbSC steady state x HbSS steady state  
   p-value = <0.001
b. HbSCvoc x HbSS steady state  
   p-value = <0.001
c. HbAA x HbSS steady state  
   p-value = <0.001
d. HbSC steady state x HbSSvoc  
   p-value = <0.001
e. HbSCvoc x HbSSvoc  
   p-value = 0.0101
f. HbAA x HbSS_{VOC}  
   p-value = <0.001
g. HbAA x HbSC steady state  
   p-value = <0.001
h. HbAA x HbSCvoc  
   p-value = 0.0031
IV: HCT

a. HbSC steady state x HbSS steady state  p-value = <0.001
b. HbSC\textsubscript{VOC} x HbSS steady state  p-value = <0.001
c. HbAA x HbSS steady state  p-value = <0.001
d. HbSC steady state x HbSSvoc  p-value = <0.001
e. HbSCvoc x HbSSvoc  p-value = <0.001
f. HbAA x HbSSvoc  p-value = <0.001
g. HbAA x HbSC steady state  p-value = <0.001
h. HbAA x HbSC\textsubscript{voc}  p-value = 0.0010

V: MCV

a. HbSSvoc x HbSC steady state  p-value = 0.0166
b. HbSS steady state x HbSC steady state  p-value = <0.001
c. HbSS steady state x HbSSvoc  p-value = 0.0139
d. HbSS steady state x HbAA  p-value = <0.001

VI: MCHC

a. HbSC steady state x HbSSvoc  p-value = 0.0390
b. HbSC steady state x HbSS steady state  p-value = 0.0425
VII: RDW

a. HbSSvoc x HbSCvoc p-value = 0.0196
b. HbSS steady state x HbSCvoc p-value = 0.0341
c. HbAA x HbSCvoc p-value = 0.0015
d. HbSSvoc x HbSC steady state p-value = 0.0038
e. HbSS steady state x HbSC steady state p-value = <0.001
f. HbAA x HbSC steady state p-value = <0.001

VIII: PLT

a. HbSC steady state x HbAA p-value = 0.0193
b. HbSSvoc x HbAA p-value = <0.001
c. HbSS steady state x HbAA p-value = <0.001
d. HbSSvoc x HbSCvoc p-value = <0.001
e. HbSS steady state x HbSCvoc p-value = <0.001
f. HbSSvoc x HbSC steady state p-value = <0.001
g. HbSS steady state x HbSC steady state p-value = <0.001

VIX: MPV

a. HbSSvoc x HbSS steady state p-value = 0.0133
b. HbSCvoc x HbSS steady state p-value = 0.0022
c. HbSC steady state x HbSS steady state p-value = <0.001
d. HbAA x HbSS steady state p-value = <0.001
e. HbSC steady state x HbSSvoc p-value = 0.0093
f. HbAA x HbSS steady state p-value = <0.001
g. HbSC steady state  x  HbSSvoc  
   p-value = <0.001

h. HbAA  x  HbSSvoc  
   p-value = 0.0401

**X: PCT**

a. HbSS steady state  x  HbAA  
   p-value = 0.0134

b. HbSS steady state  x  HbAA  
   p-value = <0.001

c. HbSSvoc  x  HbAA  
   p-value = <0.001

d. HbSS steady state  x  HbSCvoc  
   p-value = 0.0015

e. HbSSvoc  x  HbSCvoc  
   p-value = <0.001

f. HbSS steady state  x  HbSC  steady state  
   p-value = <0.001

g. HbSSvoc  x  HbSC steady state  
   p-value = <0.001

**XI: PDW**

a. HbSSvoc  x  HbSC steady state  
   p-value = 0.0185

b. HbSS steady state  x  HbSC  steady state  
   p-value = <0.001

c. HbAA  x  HbSC steady state  
   p-value = <0.001

d. HbSS steady state  x  HbSCvoc  
   p-value = 0.0285

e. HbAA  x  HbSCvoc  
   p-value = <0.001

f. HbAA  x  HbSSvoc  
   p-value = 0.0014